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Effects of Fluctuating, Sublethal Applications of Heavy Metal Solutions Upon the Gill Ventilation Response of Bluegills (Lepomis macrochirus)

Virginia Polytechnic Inst. and State Univ.
Blacksburg

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The ventilatory response of the bluegill to fluctuating, sublethal amounts of heavy metals was investigated. Non-contact submerged, stainless steel electrodes were used to detect the weak electrical potentials that are produced when fish ventilate their gills. These signals were amplified using high-gain amplifiers which were interfeced with a minicomputer. These ventilatory data were accumulated continuously, and both the ventilatory rates and average signal amplitudes were recorded on electronic tape for later analysis.

The response to these toxic solutions was an increase in rate as well as a decrease in signal amplitude. The latter response was shown to be a real response of the fish and not due to changes in the electrical properties of the water when toxicant was added. Due to the extreme smoothing of the data, the analysis of variance was unable to detect any differences between the toxicant application patterns. It was significant to note, however, that the fish were capable of reacting to each subsequent pulse of toxicant in a similar manner.

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EFFECTS OF FLUCTUATING, SUBLETHAL APPLICATIONS OF HEAVY METAL SOLUTIONS UPON THE GILL VENTILATION RESPONSE OF BLUEGILLS (*Lepomis macrochirus*)

by

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FOREWARD

The Federal Water Pollution Control Amendments of 1972, the 1977 Clean Water Act, and the Toxic Substances Act all require control of the discharge of toxic substances. A reasonable data base exists on toxicity of known chemical compounds to aquatic organisms. However, information is limited concerning the toxicity or potential hazard of complex wastes that contain numerous chemicals in varying concentrations and combinations. Because of the increasing number and complexity of chemical compounds being manufactured and the fact that aquatic organisms are not usually exposed to individual chemicals, but rather to mixtures of several or more, it is obvious that we cannot control toxic discharges on a chemical-by-chemical basis. Thus, a rapid biological test method is needed by regulatory agencies and dischargers to evaluate the potential hazards of complex and variable wastes prior to discharge into receiving waters. The biological system must integrate the effects of these complex mixtures over time.

This report provides information concerning the acute toxicity of several known heavy metals as well as mixtures of these toxicants. The report also provides information regarding the use of fish respiratory impairment as a measure of toxicant stress when subjected to sublethal fluctuating concentrations of individual heavy metals and mixtures of these toxicants.

William B. Horning
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PREFACE

The original proposal, forwarded January 28, 1977, listed John Cairns, Jr. as the principal investigator. Kenneth L. Dickson and W. H. van der Schalie were to be staff members on the project. Van der Schalie left before the project was funded and was replaced by Kenneth W. Thompson when the project was funded. Dickson's responsibilities were taken over by Albert C. Hendricks in June, 1978.

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ABSTRACT

The ventilatory response of the bluegill to fluctuating, sublethal amounts of heavy metals was investigated. Non-contact, submerged, stainless steel electrodes were used to detect the weak electrical potentials that are produced when fish ventilate their gills. These signals were amplified using high-gain amplifiers which were interfaced with a minicomputer. These ventilatory data were accumulated continuously, and both the ventilatory rates and average signal amplitudes were recorded on electronic tape for later analysis.

Acute toxicity of each of the heavy metal solutions used was determined as well as the various mixtures used. The compounds used and the LC50s determined (in parentheses) were $ZnSO_4$ (3.2 mg/l), $CuCl_2$ (1.0 mg/l), $NiCl_2$ (21.2 mg/l), and K_2CrO_4 (132.9 mg/l). The toxicity of zinc-copper and zinc-copper-nickel-chromium mixtures was found to be additive under the test conditions.

Use of conventional parametric procedures is inappropriate for analysis of ventilatory data from an individual fish as successive observations are not independent. This report determined that such analyses are appropriate when comparing data between individuals.

The ventilatory behavior of bluegills (*Lepomis macrochirus*) receiving sublethal exposures of heavy metal solutions was compared to unexposed controls. The toxicant solutions were delivered in short, intermittent 5-hour pulses, longer 20-hour pulses, and conventional 96-hour applications.

The response to these toxic solutions was an increase in rate as well as a decrease in signal amplitude. The latter response was shown to be a real response of the fish and not due to changes in the electrical properties of the water when toxicant was added. Due to the extreme smoothing of the data, the analysis of variance was unable to detect any differences between the toxicant application patterns. It was significant to note, however, that the fish were capable of reacting to each subsequent pulse of toxicant in a similar manner.

Both ventilatory rate and ventilatory amplitude, especially when used in conjunction, can be powerful tools for the continuous monitoring of surface water quality.

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SECTION 1

INTRODUCTION

The increasing volume and complexity of industrial, agricultural, and other wastewater effluents in the modern world make imperative the development of rapid methods for early detection of developing toxic situations. Use of a living organism as a generalized monitor capable of responding to the entire toxicant load of its environment has been suggested by numerous authors (see Cairns and van der Schalie, in press, for a review). A primary requirement for such a living monitor would be a detectable response to sublethal conditions as a warning of the developing lethal situation. Many physiological and behavioral parameters might be used as indicators of stress situations. One that shows considerable promise and has received attention by many workers is the rate at which a fish passes water across its gills during "breathing" activity (ventilation).

The ventilatory rates of fishes have been used as an indicator of stress for many years, and numerous methods have been employed for observing the ventilatory process. The oldest and simplest method is visual counting of the opercular movements, a method that has proven useful for short-term studies for many years (Belding, 1929; Meuwis and Heuts, 1957; Chizar et al., 1972; Gee et al., 1977; Sorensen and Burkett, 1978).

Many workers have measured ventilatory rates with surgically attached or implanted sensors of various types (Saunders, 1961; Pasztor and Kleerekoper, 1962; Schaumburg et al., 1967; Cairns et al., 1970; Davis, 1972; Sparks et al., 1972; Young, 1971; Hughs, 1975; Lunn et al., 1975; Sellers et al., 1975; Barnett and Toews, 1978). These attachments may contribute to the stress of the fish, and the necessity of making these attachments, complicated or not, to each individual specimen precludes the use of such methods for gathering large, long-term data sets from large numbers of individuals and species. Such data sets are an absolute necessity if any validity is to be attached to predictions made with such information.

The most recently developed method for determining the ventilatory rates of fishes is also the one that lends itself best to the accumulation of a large data base from numerous individuals and species. This method detects, by submerged, noncontact electrodes in aquaria, the weak electrical signals

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produced by the movement of the muscles in the branchial region as a fish ventilates the gills during "breathing" activity (Figures 1 and 2).

Possibly the earliest workers to use such noncontact sensing were Kleerekoper and Sibakin (1956) who investigated the weak electrical signals produced by the lamprey; however, their subject was physically restrained in a plastic tube. Camougis (1960) used noncontact electrodes to sense the bioelectrical potentials of fish and other aquatic animals that were unrestrained in an aquarium, and thus presumably, were less stressed than when restrained. Probably the earliest use of this type of apparatus to describe the response of freshwater fish to environmental variables was that of Roberts (1964) who studied the effects of temperature and photoperiod on Lepomis gibbosus. This type of apparatus has been used more recently to investigate ventilatory response to various environmental contaminants (Spoor et al., 1971; Drummond et al., 1973; Drummond et al., 1974; Thomas and Rice, 1975; Drummond and Carlson, 1977; Carlson and Drummond, 1978; Maki, 1979; Thomas and Rice, 1979). While some of these investigators reported the effects on ventilatory rates, the majority emphasized the cough or "gill purge" frequencies with little emphasis on actual ventilatory rates or their changes. Most noncontact studies mentioned thus far have relied on the analysis of short intermittent segments of strip chart recordings which leave a large amount of the ventilatory behavior undescribed.

Recent technological advances in the field of electronics have made relatively low-cost, dependable minicomputers, microprocessors, or other electronic recording devices economically feasible for laboratory use. These devices are easily adaptable for the continuous accumulation of fish ventilatory data and make possible the establishment of the large data bases necessary for statistical predictions and valid long-term environmental monitoring. Results from such systems have been reported by Morgan and Kuhn (1974), Morgan (1977), Westlake and van der Schalie (1977), Thompson et al. (1978), Morgan et al. (1979), van der Schalie et al. (1979), and Cairns and Thompson (in press).

Most toxicity data are based on conventional tests in which constant toxicant concentrations are maintained and in which the time to death of the test organism is the measured parameter. However, fluctuating conditions are the rule in the "real world" and constancy the exception. In order to develop a living, continually sensing, water quality monitor, controlled "real world" conditions must be simulated in the laboratory for determining predictable stress responses. The non-stressed behavior of the test organism must first be described for comparison.

The present study was instigated to investigate and describe the ventilatory response of the bluegill (Lepomis macrochirus) to simulated "real world" conditions (that is, to fluctuating,

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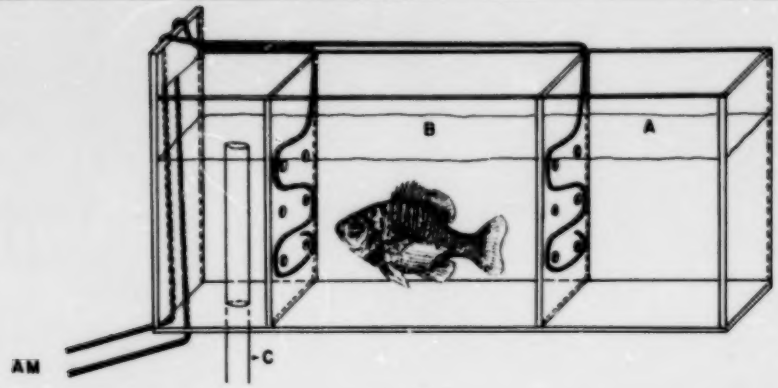

Figure 1. A bluegill in a ventilatory sensing chamber. Water flows into the receiving chamber (A), through the sensing chamber (B), and out the drain (C). The electrodes are the S-shaped wires at either end of the sensing chamber. The signal is carried to an amplifier (AM).

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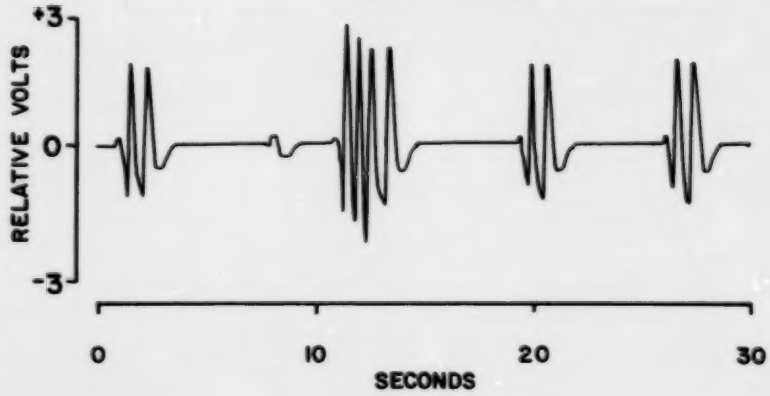

Figure 2. An example of the amplified ventilatory signal of a bluegill ventilating at approximately 20 times per minute.

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complex, sublethal toxicant concentrations) and to accumulate a large enough data base to allow valid statistical characterization and analyses. The toxicants used are all common components of industrial effluents. Standard 96-hr median lethal concentrations under conditions in this laboratory were established for all the toxicants used in order to relate to conventional toxicity data and determine the concentrations to be used in the ventilatory studies.

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SECTION 2

CONCLUSIONS

1. Ninety-six hour median lethal concentrations for divalent zinc, copper, and nickel, and for hexavalent chromium were determined to be 3.2, 1.0, 21.2, and 132.9 mg/l, respectively.

2. At the concentrations tested, the toxicities of mixtures of zinc and copper and of zinc, copper, nickel, and chromium were determined to be additive.

3. The ventilatory response of the bluegill (*Lepomis macrochirus*) was shown to be measurably affected by sublethal concentrations of zinc, copper, and the complex mixtures indicated above. This effect can be used as an indication of stress in an automated biological monitor.

4. Ventilatory data meet the requirements for parametric statistical analyses when comparisons are between fish and, thus, when sample size is large enough, can be analyzed by conventional methods.

5. The bluegill displays two sensitive ventilatory responses to sublethal amounts of heavy metals: the ventilatory rate increases and the amplitude of the ventilatory signal decreases. Both of these changes are statistically detectable and are reversible on removal of the toxicant within the limits of this study.

6. Under the conditions of these tests, the addition of the heavy metal compounds could account for only a small portion of the reduction in signal amplitude indicating that the observed reduction was primarily due to a real change in the ventilatory behavior of the fish.

7. No statistical differences could be demonstrated in the average ventilatory response with respect to the pattern in which the toxicant was applied. However, graphic results indicated that the fish tend to respond to each successive toxicant application in a similar manner, even when only 5 hours recovery time was allowed.

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SECTION 3

MATERIALS AND METHODS

TEST SPECIMENS

The test organism was the bluegill, Lepomis macrochirus. All specimens used during this study were obtained from Osage Catfisheries, Inc. of Osage Beach, MO and were maintained in 450-ℓ continuous flow aquaria. The light regimen was continuous light, and the temperature was maintained at a constant 22.5±1°C. These conditions were maintained for both acute toxicity and ventilatory studies.

Acclimation and Health

The fish were acclimated to these holding conditions for at least 2 weeks before being used for toxicity tests and for 4 to 6 weeks, and often as long as six months or more, before being used for ventilatory studies.

All fish were free of any visible disease (mortality rate = 0) for at least 2 weeks before being used for either toxicity or ventilatory studies. Newly acquired specimens were given routine prophylactic treatment with erythromycin (Maracyn), minocycline (Maracyn 2), and sodium chloride. Losses due to handling damage and subsequent bacterial infections were heavy prior to instigating these treatments but dropped to near zero after they were started. Occasional infections of Ichthyophthirius (Ich) were treated with malachite green (0.7 percent solution by weight, dose = 0.04 ml/ℓ).

Specimen Size

The average standard length of the fish used in the toxicity tests was 50.1 mm with a range of 28 - 68 mm. This was determined by measuring 20 individuals selected indiscriminately from each test. The standard length of all individuals used in the ventilatory studies was measured at the end of each run. The average was 57.8 mm with a range of 49 - 74 mm. The average wet weight of the fish used in the ventilatory studies was 6.8 g with a range of 3.2-9.6 g.

TOXICITY TESTS

Two standard 96-hr continuous flow toxicity tests were run for each individual toxicant and for each toxicant mixture.

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Proportional Diluter

A solenoid valve controlled proportional diluter system similar to that described by Hendricks et al. (1977) and Peltier (1978) was used for the toxicity tests. In this system, six fish holding chambers of 6.4 ℓ capacity received 233 ml of solution every 3 min providing a flow rate of 4.66 ℓ per hour (Figure 3). This rate was equivalent to 17.5 chamber volumes during each 24-hr period.

The head boxes, proportioning boxes, mixing chambers, and fish holding chambers were constructed of 0.64 cm plexiglas with methylene chloride fused joints. Delivery tubes and standpipes were of teflon, and fittings were of glass. The toxicant reservoir was polyethylene (Figure 3). The diluter was scrubbed out and then flushed with a solution of ethylenediamine tetraacetic acid (EDTA) between toxicity tests.

The toxicant solutions were delivered to the mixing head box by a variable speed peristaltic pump. The concentration of toxicant in the fish chambers could be changed between tests by one or a combination of the following four methods: the concentration of the toxicant in the toxicant reservoir could be changed, the flow rates of the diluent water or of the toxicant solution could be changed, or the percentage of toxicant could be adjusted. The latter could be accomplished by changing the height of the adjustable standpipes in each compartment of the proportioning boxes (Figure 3).

Protocol for Toxicity Tests

Ten fish were placed in each of the fish chambers at the start of each toxicity test. The number of individuals surviving in each chamber was recorded after 1, 2, 4, 8, 12, and 24 hr and every 24 hr thereafter. All dead fish were removed at each observation. Death was defined as the loss of equilibrium. Fish that could not respond to prodding with a blunt rod by righting themselves were considered dead.

Probit Analysis

Probit analysis as described by Finney (1971) was performed to determine the 96-hr median lethal concentration (LC50) values and their 95 percent confidence limits. A computer program available on SAS79 was used to carry out this analysis (SAS Institute, 1979).

Two toxicity tests were run with each toxicant solution. Toxicant concentrations were adjusted slightly when necessary for the second test to allow for better resolution at concentrations near the LC50 since probit analyses are more meaningful

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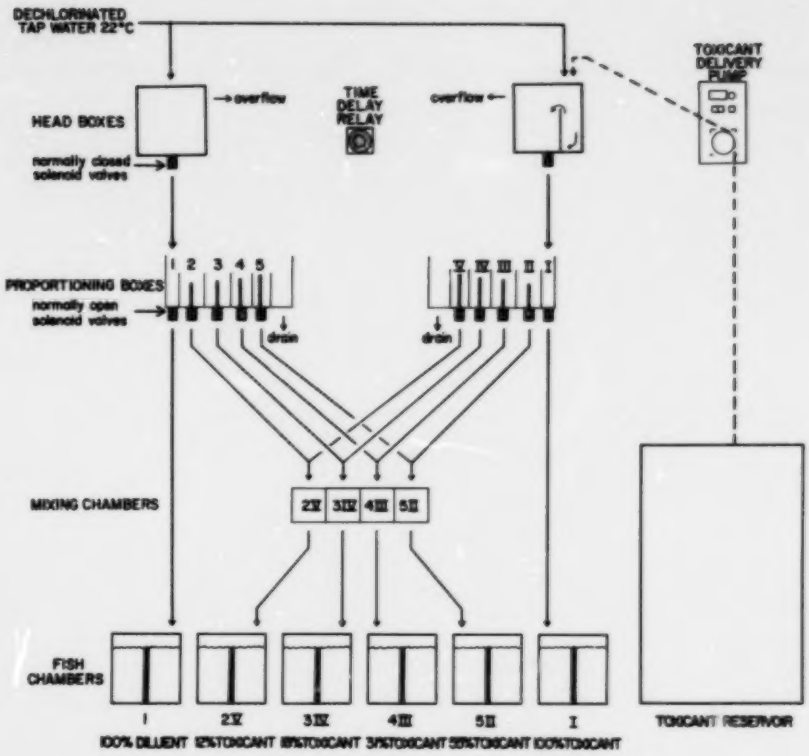

Figure 3. The solenoid controlled, continuous flow, proportional diluter. The time delay relay simultaneously opens the valves on the head boxes and closes those on the proportioning boxes allowing the proportioning boxes to fill with diluent and toxicant solutions. When the level of the liquid in the diluent proportioning box reaches the top in compartment 5, a float valve is tripped and the solenoid valves are returned to their normal state, delivering toxicant and diluent first to the mixing chambers and ultimately to the fish chambers. This also resets the time delay relay, and the cycle is repeated.

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with mortalities other than 100 percent or 0 percent. The results of successive tests with the same toxicants were in fairly close agreement, and the results of tests using the same toxicant were pooled to increase sample size. The probit analysis was run on the pooled data.

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Joint Toxicity

Since organisms may be more sensitive to some toxicants than to others, a method of equating the toxicity of the various components of a mixture was needed. Sprague (1973) recommended that the concentration of a toxicant in a mixture be reported as a proportion of the LC50 as determined singly. In this method, the sum of the ratios for all components of a toxicant mixture is determined. If this total is 1.0, the toxicity of the mixture is considered to be additive. A total of less than 1.0 is considered to be synergistic (greater than additive), and a total greater than 1.0 is considered to represent a mixture with antagonistic (less than additive) toxicity. Marking (1977) used this concept as a basis for the development of a linear additive index in which values of zero indicate additive toxicity, positive values indicate synergistic toxicity, and negative values indicate antagonistic mixtures. Marking's additive index (MAI) has the advantage of providing a method for establishing critical limits for values that are near zero.

MAI can be calculated using the equation:

$$\sum_{1}^{i} A_{mi}/A_i = S$$

where A_{mi}/A_i = toxic unit, i = the total number of toxic components in the mixture, A_{mi} = LC50 for a toxicant in the mixture, A_i = LC50 for that toxicant as determined individually, and S = the sum of the biological effects or the sum of the toxic units. Then MAI = (1/S) - 1.0 if $S \leq 1.0$, and MAI = 1.0 - S if $S \geq 1.0$.

The significance of the deviation from zero can be determined by substituting values of the 95 percent confidence limits for the LC50 values of both A_i and A_{mi} into the formula for MAI to establish a range for MAI. The values that yield the greatest deviation from MAI are used to establish the range. If the range includes zero, the deviation is not considered to be significant.

WATER CHARACTERISTICS

Dechlorinated tap water from a single source was used as diluent water for all toxicity tests and ventilatory studies and to supply the holding tanks.

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Temperature

Water was maintained at a constant temperature with a Thermatreck 34-E temperature controller. A malfunction of the temperature controller during the first toxicity test using copper resulted in a temperature of 19°C ± 2.5°C. In all other toxicity tests and all ventilatory studies, the temperature was maintained at 22.5°C ± 1°C. A Tempscribe remote bulb temperature recorder was used to monitor the temperature of the diluent water continuously. Additionally, daily temperature measurements were taken from each fish chamber of the proportional diluter when toxicity tests were being run.

Water Chemistry

Dissolved oxygen, conductivity, pH, alkalinity, and total hardness were measured in each of the six fish chambers at the beginning of every toxicant test and once during each subsequent 24-hr period for the duration of a test. Consequently, five measurements were taken from each chamber during the tests. Since the diluent water for both toxicity testing and ventilatory studies was from the same source, chemical data were not measured for the ventilatory chambers. The chemical values of the diluent water in the ventilatory studies is assumed to be the same as those measured from chamber 1 (control) of the toxicity tests.

Dissolved oxygen was measured with a YSI Model 54 A oxygen meter. Conductivity in micromhos/cm was measured with a Beckman Model SM-210 conductivity meter modified to read in the 0 - 500 micromho range. When concentrations of toxicant were high enough to cause values greater than 500, the samples were diluted with distilled water (conductivity = 0 micromho/cm). A Corning Model 7 pH meter was used to determine the pH. Alkalinity and total hardness were determined titrimetrically according to methods described by American Public Health Association (APHA) et al. (1976). Total hardness could not be determined in this manner when nickel was present and was determined instead by measuring the calcium and magnesium concentrations by atomic absorption spectrophotometry and calculating total hardness by the methods given by APHA et al. (1976).

The dissolved oxygen concentration was maintained at levels above 6.6 mg/ℓ in all fish chambers throughout the toxicity testing. Dissolved oxygen during ventilatory studies was measured only at the end of a test and averaged 7.7 mg/ℓ.

Conductivity of the diluent water ranged from 100 to 145 micromhos/cm, pH from 6.7 to 7.6, alkalinity from 22 to 42 mg/ℓ, and total hardness from 16 to 82 mg/ℓ. High concentrations of toxicants, especially of hexavalent chromium caused the conductivity to go as high as 3352 micromhos/cm, the pH to reach 8.3, and the alkalinity to reach 564 mg/ℓ. The average values for

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each fish chamber during each toxicity test are given in Appendix A (Table A-1).

A Wallace and Tiernan Amperometric Titrator was used to periodically monitor the total chlorine content of the diluent water. The total chlorine level remained between 0.01 and 0.05 mg/l throughout the course of these studies.

Toxicant Measurement

Toxicant concentrations, as mg/ℓ of the metal ion, were determined with a Perkin-Elmer model 460 atomic absorption spectrophotometer. Analyses were done using an air-acetylene flame except when both nickel and chromium were present. In that case a nitrous oxide-acetylene flame was used for the determination of the concentration of the hexavalent chromium since nickel blocks the detection of chromium. Water samples for determination of the concentration of the toxicant were acidified with concentrated nitric acid, sealed with parafilm, and held until the end of a test before being analyzed.

During toxicity testing, five water samples were taken as indicated for water chemistry. During ventilatory studies, water samples were collected below the drain without disturbing the fish. These samples were taken when appropriate depending on the toxicant application pattern. This was arranged so at least four samples were taken at each toxicant level, and at least one sample before and one after the toxicant was applied. While each sample was analyzed separately the average concentration for each fish chamber or ventilatory sensing chamber was used for final analyses.

Toxicant Solutions

The toxicant sources were reagent grade zinc sulfate ($ZnSO_4 \cdot 7H_2O$), cupric chloride ($CuCl_2 \cdot 2H_2O$), nickelous chloride ($NiCl_2 \cdot 6H_2O$), and potassium chromate (K_2CrO_4). To provide divalent zinc, copper, nickel, and hexavalent chromium these compounds were dissolved in distilled water. When chromium was one of the metals in a toxicity test, an airstone was placed in the mixing head box of the proportional diluter to facilitate mixing. The chromium solution was so concentrated that a density gradient between the diluent water and the chromium solution formed and resulted in a very concentrated layer of toxicant solution in the bottom of the mixing box. However, no precipitate was observed in the mixing head box.

When determining the toxicity of complex solutions, it is desirable to control the ratios of the toxic strength of the various toxicants so that one component is not the major contributor of toxicity. Tsai and McKee (1980) defined the toxicity ratio in the following manner:

$$\text{Toxicity ratio} = A_{m1}/A_1 : A_{m2}/A_2 \ldots A_{mi}/A_2 \quad (2)$$

where the definitions of the terms are the same as for equation (1)

Due to interactions between the dissociation products of the various chemical compounds and between these products and the diluent water which resulted in various precipitates, separate solutions of each compounds for use in joint toxicity tests were prepared. Each was then pumped separately into the toxicant head box where mixing occurred (Figure 3). Because of these complications, the desired toxicity ratio of 1:1 was achieved only for MixA (Zn^{++}, Cu^{++}). The toxicity ratio ultimately achieved for MixB (Zn^{++}, Cu^{++}, Ni^{++}, Cr^{+6}) was approximately 1:1.8:1.7:1.6. However, since the primary purpose of these toxicity tests was to indicate concentrations in the sublethal range for use in ventilatory studies, these results were accepted and no further toxicity tests were carried out.

VENTILATORY STUDIES

The electrical action potential of muscle movement in the branchial region of a fish during ventilatory or "breathing" activity produces an electronic signal. A system for recording this signal continuously and for applying toxicants to the fish in varying, pre-selected patterns was constructed.

Sensing Chambers

A fish ventilatory sensing chamber with a total of 16 individual 700 ml capacity monitoring compartments was constructed of 6.35 mm transparent plexiglas. The ends and dividing baffles were of 3.18 mm transparent plexiglas, and the sides of each individual compartment were of 3.18 mm opaque white plexiglas (Figures 4 and 5). All joints were fused with methylene chloride.

Electrodes

The submerged electrodes were of type 304 stainless steel wire and were attached with silicone aquarium cement to the baffles at either end of the monitoring compartment. The electrodes were on the side of the dividing baffles opposite the fish to prevent contact between fish and electrode. Each electrode was formed in such a way that the wire never passed through the flow of water from any of the perforations in the dividing baffles (Figure 4). The electrode wire was insulated from just below the water line, where the insulation was sealed to the wire with silicone cement to prevent water intrusion, to the barrier strip on the outside of the tank. The only exposed wire was below the water surface and at the barrier strip connections.

Isolation Chamber

Since fish respond to visual stimuli and to vibrations in

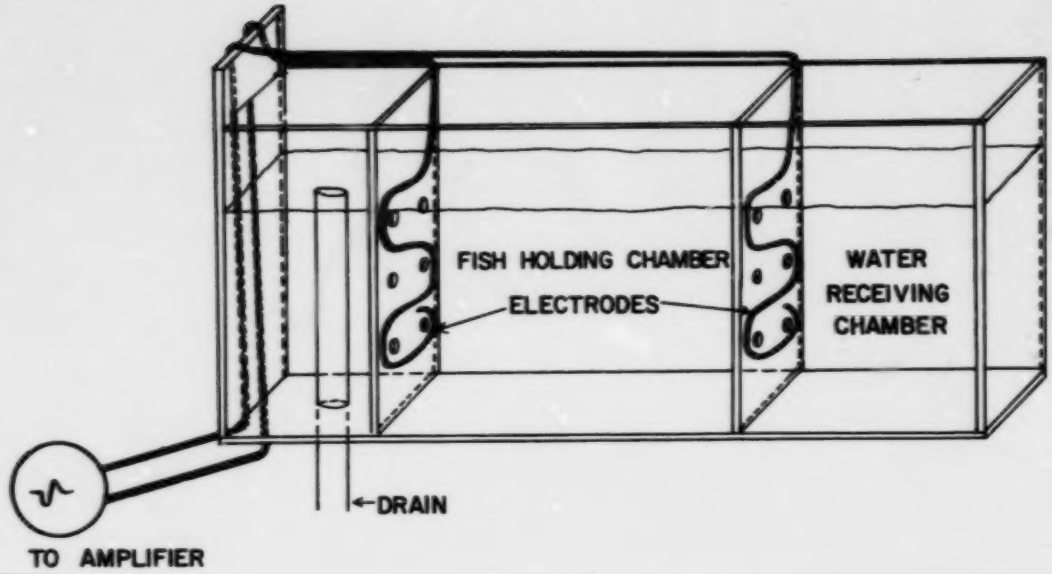

Figure 4. An individual ventilatory sensing chamber. The dimensions of the fish holding chamber are: length, 12 cm; width, 4 cm; height, 10 cm; and water depth, 6 cm.

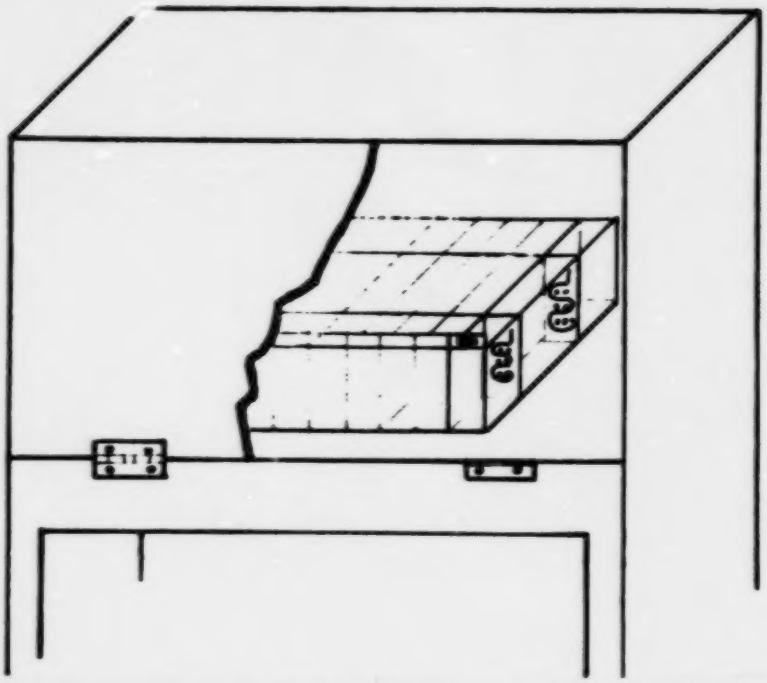

Figure 5. Cutaway view of the plywood isolation chamber to show the composite ventilatory sensing chambers.

a manner similar to their response to chemical stimuli, the entire fish holding chamber was enclosed in a plywood isolation chamber. The supports of this enclosure were anchored in sand filled containers to protect against vibration (Figures 5 and 6).

Water Delivery

Diluent water was delivered by gravity flow to three plexiglas mixing chambers where the diluent could be mixed with various toxicant solutions as desired (Figure 6). The water then was passed into delivery chambers which were also of plexiglas and equipped with 1 mm diameter standpipes. A constant head pressure was maintained over these standpipes by delivery in excess with the excess drained through an adjustable standpipe. This allowed delivery of water or water and toxicant at a constant rate and concentration to each sensing chamber (Figure 6).

Toxicant Delivery

An Intergral Masterflex, variable speed, peristaltic pump drive with remote control capability was used to introduce the toxicant into the mixing chambers (Figure 6). As many as nine pump heads could be used simultaneously for delivery of different toxicant solutions to each mixing chamber or for producing various combinations in the mixing chambers. The toxicant solutions were pumped through 1.66 mm inside diameter silicone tubing. One of the three mixing chambers was used for control and, thus, never received toxicant solution. Duplicate toxicant reservoirs, pump heads, mixing chambers, and water delivery chambers (Figure 6) allowed for replicates of the treatments during each ventilatory test. Two different treatments during a single test could have been carried out using this apparatus. Between ventilatory runs, the system was rinsed with a solution of EDTA. The minicomputer used a digital to analog converter to deliver a preselected voltage to the pump drive. A computer-pump interface was used to convert the output voltage from the computer to the optimum range for use by the pump. The computer output voltage could be changed by the computer at pre-selected 1-hr intervals to simulate fluctuating "spills" or releases. The concentration of toxicant in the individual sensing compartments reached maximum concentration in 15 - 30 minutes and dropped to backgroud level at approximately the same rate, depending on the flow rate through the compartment (Figures 7 and 8).

Amplifiers

An amplifier designed for use with fish ventilatory signals has been described in detail elsewhere (Gruber et al., 1977). Sixteen of these high-gain noise immune amplifiers, one for each of the sensing compartments, were constructed for this project. The total gain of this amplifier is variable from 29,200 to 321,000 times. Due to individual variability among test subjects,

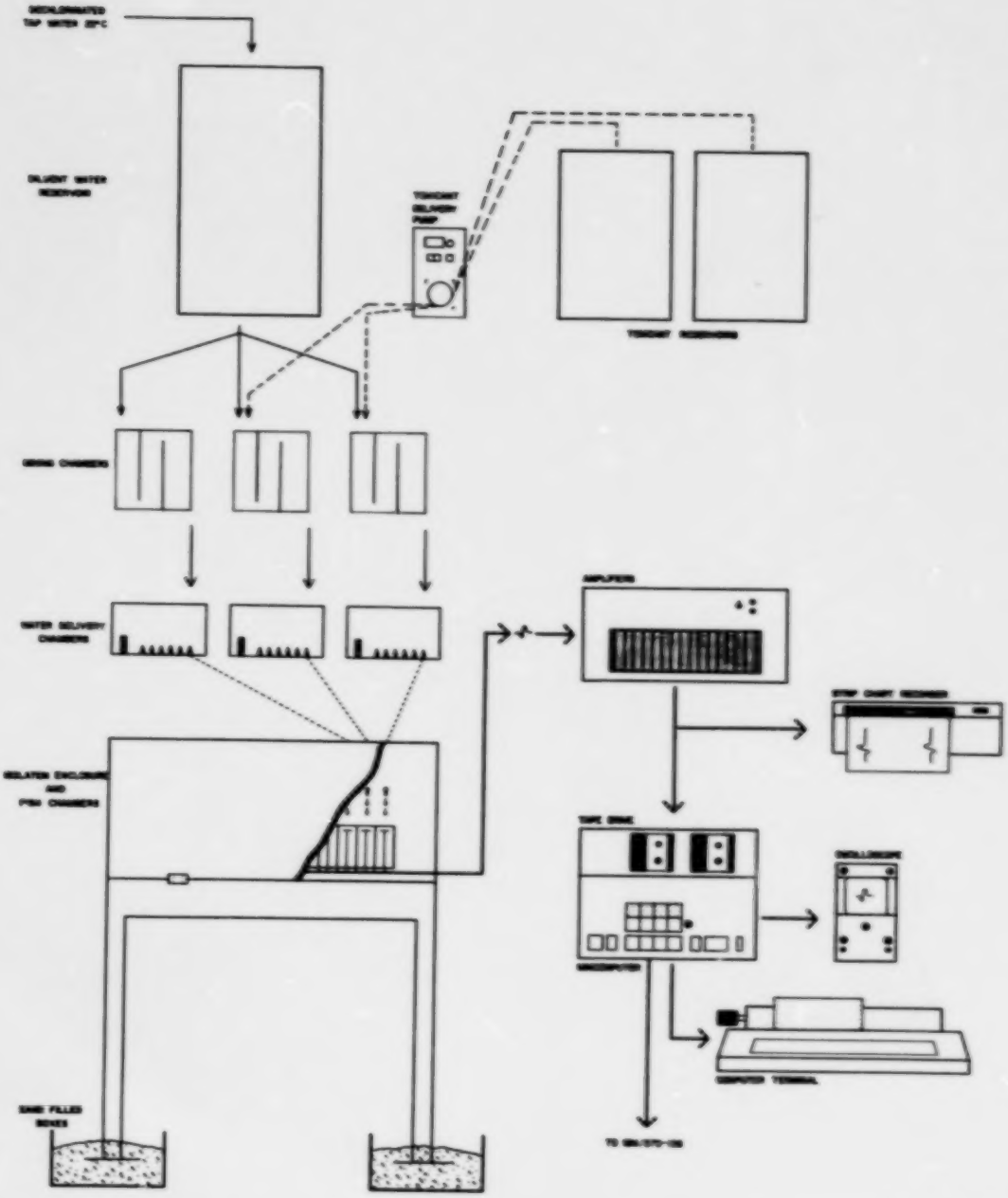

Figure 6. The water delivery and data accumulation system.

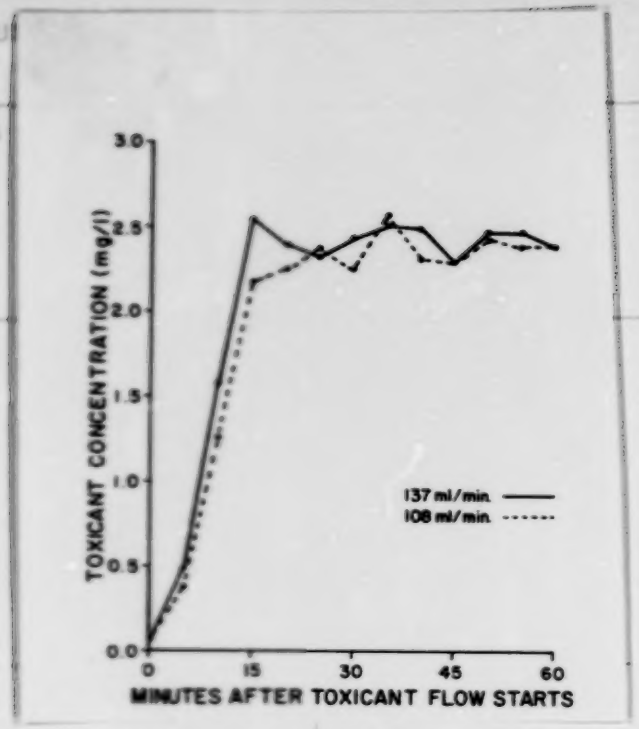

Figure 7. The rate of increase of the concentration in the ventilatory sensing chambers.

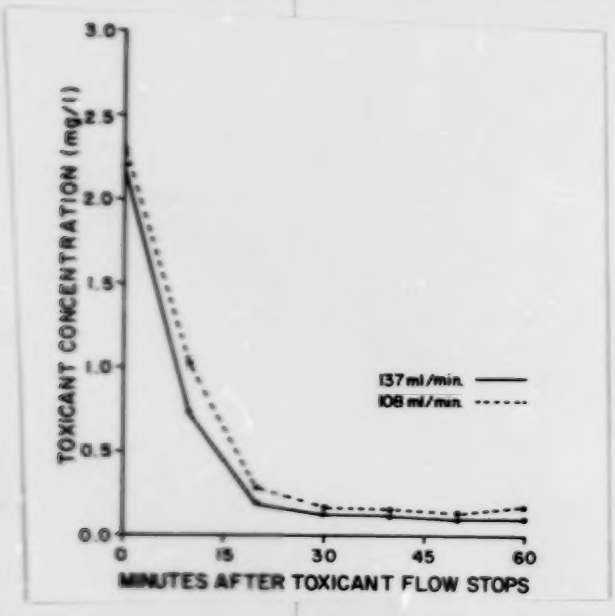

Figure 8. The rate of decrease of the concentration in the ventilatory sensing chambers.

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each amplifier was adjusted during the acclimation period to produce a ventilatory signal in the 4 to 8 volt range; consequently, comparisons of absolute signal amplitude between fish are relative.

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Design modifications that increase the maximum range of this unit for use in marine situations have been described recently by Cairns et al. (1980).

Data Accumulation

Each of the 16 ventilatory monitoring compartments was connected to an amplifier. These in turn were interfaced to a PDP-8E minicomputer through a 16-channel multiplexed analog to digital converter (Figure 6).

Ventilatory counts and an average scaling factor for each fish were accumulated for 15-min periods. The scaling factor is a value from which an approximation of the average signal amplitude can be recovered. At the end of each of the 15-min periods, the accumulated data were printed on a Decwriter II computer terminal. At 30-min intervals, the two preceeding 15-min data sets were stored on magnetic tape cassettes by a Decassette TU60 tape drive.

At the end of each ventilatory run, the minicomputer was connected by telephone line to an IBM/370-1580, and data stored on the cassettes were transcribed onto a Conversational Monitoring System (CMS; an interactive programming system for the IBM System/370). Transcription took approximately 1 hr. The minicomputer could then be reset and another ventilatory study started as desired.

Computer Program

The ventilatory signal is often bimodal (Thompson et al., 1978; Gruber et al., 1979). This bimodality is an artifact of the amplifier design and is more pronounced at slower rates (Thompson et al., 1978). The signal has been visually correlated with the ventilatory activity of *L. macrochirus*, and each waveform, bimodal or not, represents one complete ventilatory cycle (Gruber et al., 1979).

The computer program developed for this project is capable of rejecting this artifact when it is present and of accumulating the total ventilatory counts as well as an approximation of the average amplitude of the signal. This program also uses a software controlled variable threshold to reject low amplitude noise. Signals with less than 1 volt peak-to-peak amplitude were considered to be noise.

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This program used the first derivative of the ventilatory signal for determining the number of cycles or waveforms in a reporting period. In each cycle there was at least one region in which a relatively large change in the signal voltage resulted in a large peak in the derivative signal at the point of maximum change. A software Schmidt trigger was used to reduce the effect of noise on the signal. This was done by establishing symmetrical positive and negative thresholds for the derivative signal. When the derivative signal exceeded the negative threshold, the count logic was cleared. When a following positive signal exceeded the positive threshold, the logic was clocked and a count registered. Consecutive pulses of the same polarity did not produce counts unless there was a pulse of opposite polarity occurring between them. Examples of the computer produced derivative signal are shown in Figures 9 and 10. The sharp negative pulse indicates that a count has been registered and further counts will be inhibited until the logic is reset by a negative pulse.

The similarity in shape of the electronic artifact to some types of gill purge signals (van der Schalie, 1980) precludes the use of this program for detecting that response. Other types of gill purges appear as signals of higher amplitude than the associated ventilatory signals. These, as well as any body movements that produce signals meeting the above criteria, would be included as ventilatory signals.

The amplitude of the input signal was variable over time and between individual fish which limited the usefulness of the fixed thresholds. To alleviate this problem, an automatic gain control was used that kept track of the amplitude of the incoming signal for 2 sec and adjusted the scaling factor for each succeeding signal either up or down to result in a predetermined peak signal level. A maximum scaling factor prevented signals with amplitudes below a specified level (1 volt peak-to-peak) from being amplified beyond the threshold and, thus, producing false counts. This was, in effect, a variable threshold which allowed a more accurate interpretation of the ventilatory events than was possible with earlier biomonitoring programs that used fixed thresholds with no automatic gain control (Cairns et al., 1977; van der Schalie, 1977). With those programs, large reductions in amplitude would have registered as a reduction in rate. With the present program, real ventilatory signals with amplitudes of less than 1 volt peak-to-peak would also be ignored, and a possible false reduction in rate would be indicated.

The length of the time period during which the amplitude of the signal was retained was variable and was determined by the operator at the start of an experiment. This time period should be similar to the period of the wave form being observed. If it is too short, the smaller peaks may be amplified to the maximum and result in false counts. If too long, a real cycle of low amplitude would be lost if a very large peak immediately preceeded

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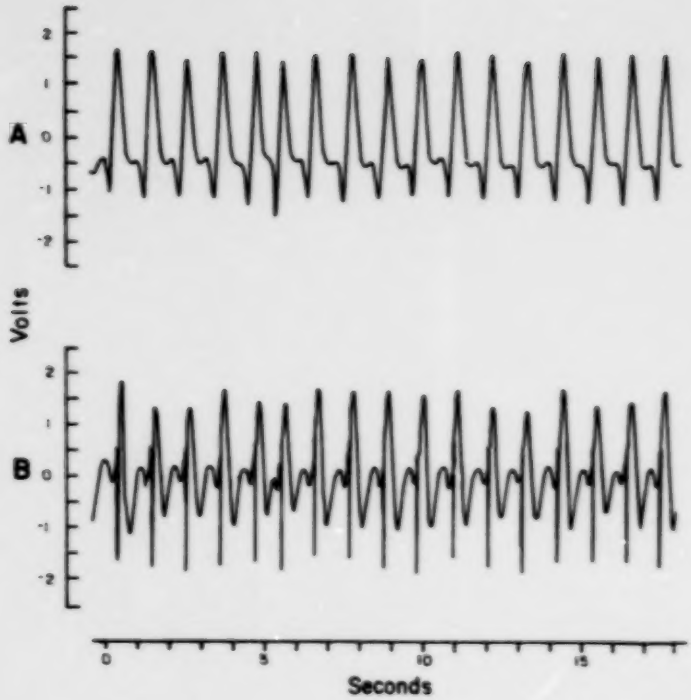

Figure 9. Strip chart recording of the ventilatory signal of a bluegill. A. The ventilatory signal as taken directly from the amplifier. B. The computer produced derivitive of the signal in A. The very sharp negative peaks indicate that a count has been recorded.

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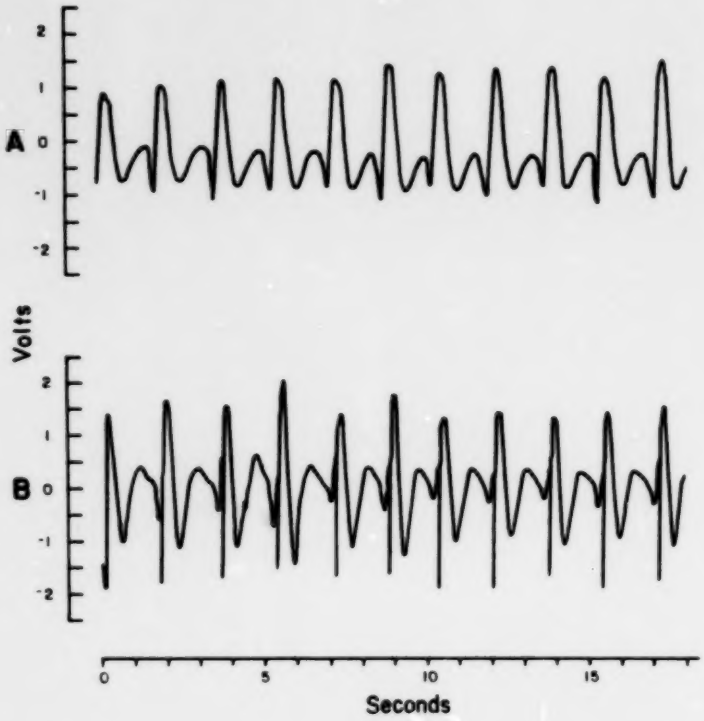

Figure 10. Strip chart recording of the ventilatory signal of a bluegill. A. The ventilatory signal as taken directly from amplifier. Note the increased bimodality with respect to Figure 9. B. The computer produced derivitive of the signal in A.

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it. Two seconds worked well for bluegills and was the value used for all of the ventilatory studies reported here.

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This program accumulates data with low error rates when in use with input signals in the optimum range of 4 to 8 volts. Normal cycles were missed less than 2 percent of the time, and extra counts were added less than 1 percent of the time. These levels remained below 10 percent even when the signals were irregular and of low amplitude.

Major Equipment Problems

DC Voltage Drift--

The fish ventilatory signal is detected as a change from zero voltage. From time to time, the background voltage drifted from zero to a positive or negative DC voltage. Detection of the signal by the computer program is dependent on the signal breaking both a positive and a negative threshold. These thresholds are set at absolute values around zero, and, if the background voltage departs greatly from zero, the signal is lost. When a sensing chamber was empty or when a fish was between breaths, this voltage should be zero in a properly adjusted system (Figure 2). A 10,000 Ω loading resistor placed across the electrodes eliminated most of the voltage drift. However, as the equipment wore out over time, the signal tended to drift from zero and data were lost (Figure 11). The results from 10 fish during the first four ventilatory studies were lost in this manner.

This problem was caused by corrosion of the terminal barrier strips which were the connectors between the electrodes and the amplifiers. These were inside the isolation chamber where the atmosphere was very humid. The problem was solved by replacing all electrodes and barrier strips. In practice, this is necessary about every 18 months under conditions of continuous use.

Electronic Noise--

While shielded cable was used for all wiring and proper grounding techniques were used, a considerable problem with electrical interference or "noise" still existed that could mask the ventilatory signals. The source of this noise appeared to be ground currents flowing from the water supply to the drain.

Water dripped into the sensing chambers from a height of 10 cm above the water surface provided a broken stream of water and eliminated this noise (Figure 6). The noise occurred only when a continuous stream of water was present to carry ground currents.

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Water Splash--

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The solution to the noise problem caused the lesser problem

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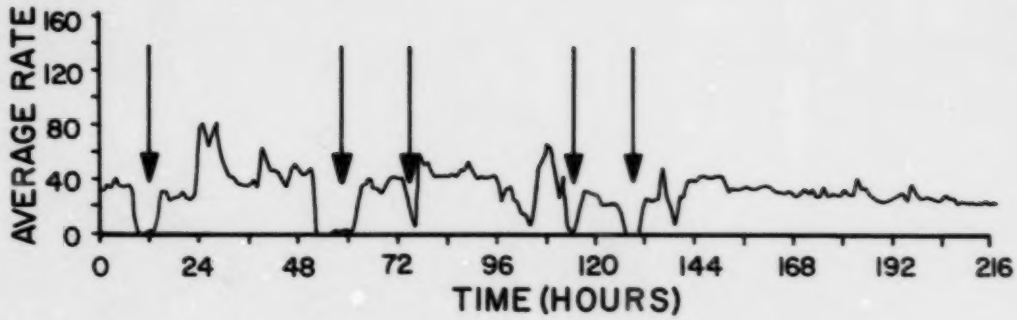

Figure 11. The average ventilatory rate of a bluegill. The arrows indicate points at which the DC voltage drift caused a loss of signal. Replacing the electrodes and terminal barrier strips eliminated this problem.

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of splash from the dripping water sources. Since controls and treated fish occupied alternate compartments, sometimes toxicant splashed into control compartments. This was a minor problem, and only one control fish was influenced by this; the data for that fish was invalidated (#6, run 6).

Ventilatory Tests

Twelve, ten-day ventilatory studies were carried out, three with each of the four toxicants. Each of the three toxicant application patterns was included among these three tests, thus all patterns were run with each toxicant (Table 1).

Toxicants--

The toxicants used for the ventilatory studies were divalent zinc, divalent copper, MixA and MixB. Neither divalent nickel nor hexavalent chromium was tested separately. All toxicants were used at concentrations that were indicated as being sublethal by the toxicity tests. The amount of toxicant solution delivered was approximately equivalent with respect to toxicity for each test with a single toxicant; however, this was not true between toxicants, and, consequently, the most valid comparisons are between patterns for each toxicant (Table 1).

Application Patterns--

In the first application pattern, toxicant was delivered to the sensing chambers at a constant level for 96 hr (Figure 12). In the second pattern, toxicant was delivered in two variable 20-hr applications separated by a 20-hr interval. The third pattern consisted of 8 variable 5-hr applications, each separated by a 5-hr interval. The 20-hour variable applications were delivered in five 4-hr increments: the first at the same concentrations as the constant 96-hr application, the second at approximately twice that concentration, and the third at three times that concentration. The concentrations then were reduced in reverse order for the fourth and fifth increments, respectively (Figure 13). The 5-hr variable applications were incremented in the same manner, but the duration of each increment was only 1 hr (Figure 14). A fourth pattern, zero, was designated for statistical analyses. Pattern zero was that of the control fish which received no toxicant.

Test Protocol--

Five of the 16 fish in each ventilatory test (3, 6, 9, 12, 15) were not exposed to toxicants and were used as controls. The remaining 11 fish were exposed to various toxicants.

Each ventilatory test was run for 102 days and consisted of 4 time intervals. Ventilatory counts and average amplitudes were

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TABLE 1. CONCENTRATIONS OF HEAVY METALS USED IN VENTILATORY STUDIES

Toxicant applications	Toxicant			
	Zn^{++}	Cu^{++}	MixA	MixB
One, 96 hr, constant	Run 1 Zn 2.5*	Run 4 Cu 0.5	Run 7 Zn 0.6 Cu 0.2	Run 10 Zn 0.3 Cu 0.1 Ni 2.5 Cr 25.5
Two, 20 hr, variable	Run 2 Zn 2.5, 5.7, 8.1	Run 5 Cu 0.5, 1.0, 1.5	Run 8 Zn 0.2, 0.4, 0.6	Run 11 Zn 0.4, 0.8, 1.1 Cu 0.1, 0.2, 0.2+ Ni 3.3, 5.1, 8.0 Cr 22.5,44.9,58.7
Eight, 5 hr, variable	Run 3 Zn 2.9, 5.7, 7.7	Run 6 Cu 0.5, 1.1, 1.3	Run 9 Zn 0.6, 1.4, 1.8 Cu 0.2, 0.5, 0.6	Run 12 Zn 0.4, 0.8, 1.1 Cu 0.1, 0.2, 0.2+ Ni 2.3, 5.3, 6.9 Cr 22.3,60.6,77.0

*All concentrations are given as the average mg/l of all fish treated during that run.

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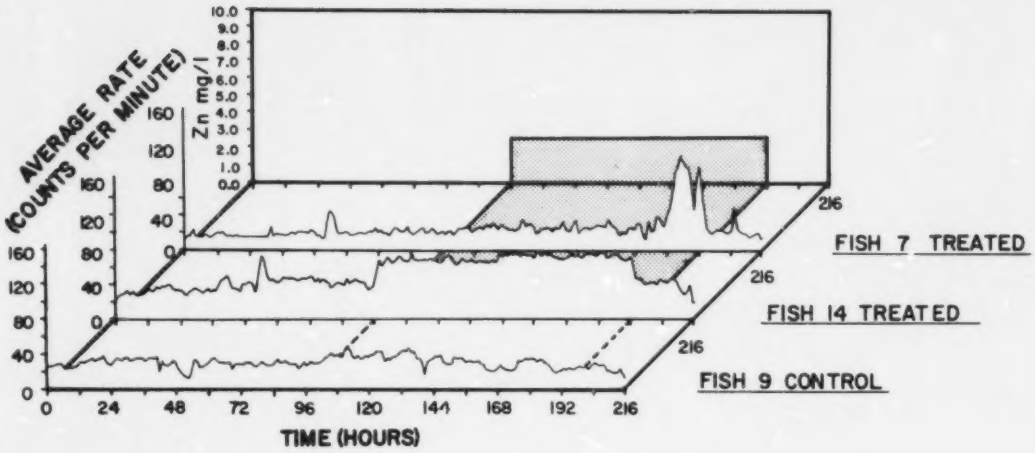

Figure 12. Toxicant pattern 1. Toxicant was delivered to fish 7 and fish 14 for 96 hr. The shaded area on the fourth vertical plane indicates the shape, duration, and concentration of the application of the toxicant. Fish 9 was a control and received no toxicant. Interval 2 = 0 - 96 hr (X axis); Interval 3 = 96 - 192 hr; Interval 4 = 192 - 216 hr. Data were not recorded during Interval 1 which was the 24 hr prior to hour 0 (see text for explanation).

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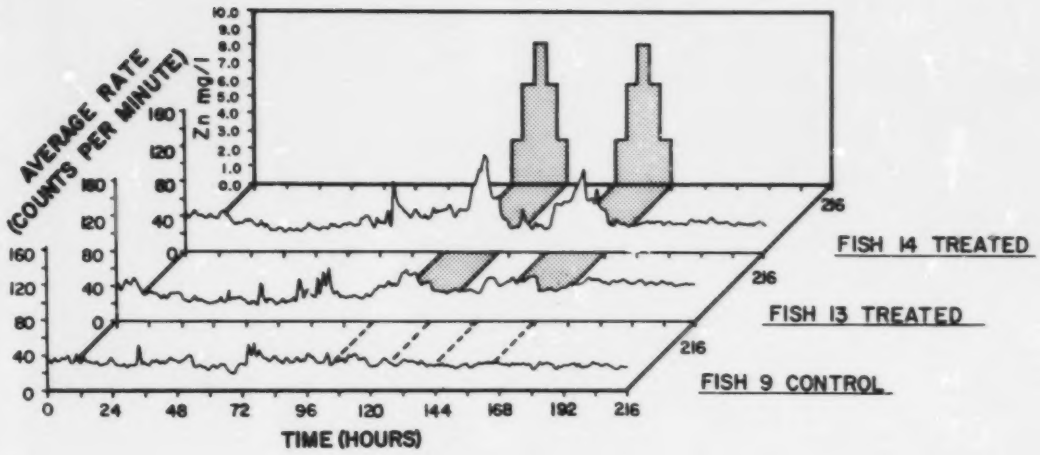

Figure 13. Toxicant pattern 2. Toxicant was delivered to fish 14 and fish 13. The shaded area on the fourth vertical plane indicates the shape, duration, and concentration of the toxicant applications. Each application lasted for 20 hr; each increment was 4 hr. Interval 2 = 0 - 96 hr (X axis); Interval 3 = 96 - 156 hr; Interval 4 = 156 - 216 hr. Data were not recorded during Interval 1 which was the 24 hr prior to hour 0 (see text for explantion).

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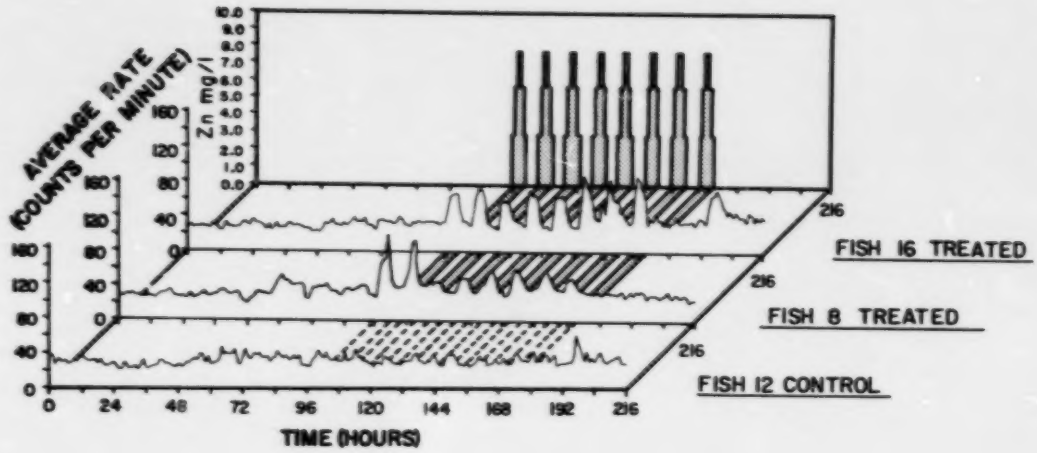

Figure 14. Toxicant pattern 3. Toxicant was delivered to fish 16 and fish 8. The shaded area on the fourth vertical plane indicates the shape, duration, and concentration of the toxicant applications. Each application lasted for 5 hr, each increment was 1 hr. Interval 2 = 0 - 96 hr (X axis); Interval 3 = 96 - 171 hr; Interval 4 = 171 - 216 hr. Data were not recorded during Interval 1 which was the 24 hr prior to hour 0 (see text for explanation).

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recorded only during the last 3 intervals, a total of 216 hr (Figures 12, 13, and 14).

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Interval 1. acclimation--The fish were placed in the sensing chambers and allowed to acclimate for 24 hr. This 24-hr period was usually sufficient time to allow stabilization of ventilatory rates (Figure 15). Amplifiers could be adjusted during this interval to produce signals in the optimum range (4 - 8 volts peak-to-peak) and, if necessary, the rate of water flow to the chambers could be reduced to eliminate electronic noise. No adjustments were permitted during the remaining three intervals.

Interval 2. pretreatment--The ventilatory rate and average amplitude were accumulated for 15-min intervals for 96 hr. No toxicant was delivered during this interval which was to provide control data for each individual fish.

Interval 3. treatment--Toxicant was delivered. This interval varied in duration depending on the pattern of application and continued only until the toxicant delivery ceased for the final time. For pattern 1, this was 96 hr; for pattern 2, 60 hr; and for pattern 3, 75 hr.

Interval 4. post-treatment--The recovery period was monitored from the end of interval 3 to the end of the ventilatory study. This was 24 hr for pattern 1; 60 hr for pattern 2; and 45 hr for pattern 3.

The duration of all intervals for pattern zero (controls) was the same as for the treated fish in each ventilatory test, respectively.

Data Treatment and Analysis

Data from four consecutive, 15-min reporting periods were summed. The ventilatory counts were then divided by 60 to yield the average counts per minute for each 1-hr period. Since the signal amplitude was reported as an average, the hourly sums were divided by 4 to produce an average voltage for each 1-hr period. Nunn et al. (in press) have shown that such ventilatory rate data are approximately normally distributed, and, thus, standard parameteric analyses performed on them are statistically valid.

These averages then were used to calculate an interval mean for each fish during each of intervals 2, 3, and 4. Thus, an average rate and amplitude were produced for each fish during the pretreatment, treatment, and post-treatment intervals. For example, the internal average rate for fish 12 (Figure 14) during the pretreatment interval was 31.0 cycles per minute; during the treatment interval, the average was 31.2 cycles per minute; and

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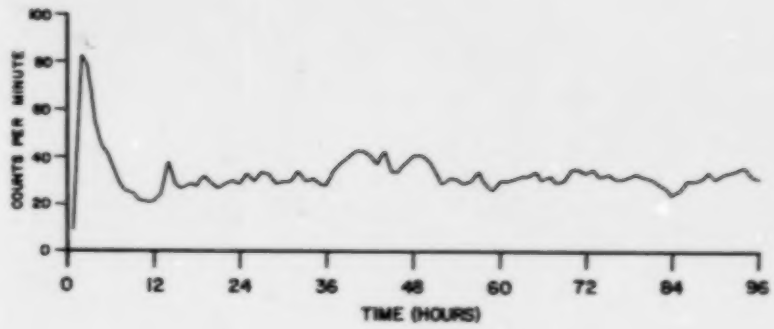

Figure 15. Average ventilatory rate for a bluegill during Interval 1 and the first three days of Interval 2. The fish was placed in the sensing chamber during the first hour.

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during the post-treatment interval 32.5 cycles per minute. These interval means were then used for statistical analyses.

Since the amounts of toxicant delivered were equivalent for each test with a single toxicant but were not equivalent between toxicants, it was decided to analyze for differences between patterns for each toxicant separately. The interval means from all ventilatory tests with a single toxicant were assembled into data sets. Each data set consisted of all the averages (interval means) from one of the intervals (2, 3, or 4) for all three tests with that toxicant. For example, the first data set was assembled from the pretreatment interval for all three runs that used Zn^{++} as the toxicant. This was then partitioned into four cells corresponding to the four patterns in which the toxicant was applied (Table 2). This was done for the ventilatory rates for each time interval for each of the 4 toxicants resulting in 12 partitioned data sets. In similar fashion, 12 data sets were assembled for the average amplitude data. Thus, each of the various analyses were performed on a total of 24 data sets. Even though replicate treatment data were available, Treatments A and B (Appendices B and C) were combined for analysis.

A chi squared test for goodness of fit was performed to compare the distribution of the interval means in each data set to the normal distribution (Siegel, 1956). Cochran's C statistic was used to test for homogeneity of variances (Winer, 1971). One-way analyses of variances for unequal sample sizes were then used to test for differences due to the pattern in which toxicant was applied. A post-mortem Duncan's Multiple Range test was then used to locate the major sources of difference (Winer, 1971). Computer programs available commercially were used to carry out the latter two analyses (SAS Institute, 1979).

In addition to the application pattern and the toxicant, four other independent variables that may have affected the ventilatory rate were measured for each individual fish. The standard length of each fish was recorded in millimeters. The average toxicity ratio was calculated by dividing the average concentration of toxicant by the LC50 of that toxicant and multiplying by the total time that the toxicant was delivered. When fluctuating applications were used, this was done for each concentration level, and the three values were summed. For MixA and MixB, the values for each individual component of the toxicant mixture were calculated and these values were summed. To determine the total dose delivered, the average toxicity ratio was multiplied by the average rate of flow through the chamber in liters per minute. The fourth variable was the maximum concentration reached (expressed in terms of toxicity ratios) during the treatment interval. For pattern 1, this was the average concentration; for patterns 2 and 3, it was the average concentration reached at the peaks of the variable applications.

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TABLE 2. EXAMPLE OF PARTITIONING OF VENTILATORY RATE DATA FOR ANALYSIS

Toxicant	Interval	Pattern 0 (control)	Pattern 1 (1 x 96 hr)	Pattern 2 (2 x 20 hr)	Pattern 3 (8 x 5 hr)
Zinc run 1	2	4 means*	9 means		
Zinc run 2	3	3 means		10 means	
Zinc run 3	4	5 means			10 means
Totals for analysis		12 means	9 means	10 means	10 means

*DC voltage drift resulted in loss of 3 fish from run 1; 3 from run 2; and 1 from run 3.

A forward stepwise, multiple regression was then used to indicate which, if any, of the independent variables, pattern, standard length, average toxicity ratio, total dose, or maximum concentration might have a significant effect on the ventilatory rate during the treatment interval. This analysis produces an F ratio to indicate the significance of each variable. An R-Square value indicates the amount of the total variability accounted for by the independent variables.

Only the data for the treated individuals from the treatment intervals were used for this analysis. Separate analyses were carried out for each of the four toxicants.

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SECTION 4

RESULTS AND DISCUSSION

TOXICITY TESTS

The results of the 96-hr continuous flow toxicity tests are presented in Table 3.

Zinc

The acute toxicity of zinc to bluegills under conditions similar to those in the present study (temperature 19 - 22.5°C in soft water) has been reported by others. All the studies used either zinc sulfate or zinc chloride as the source of zinc. Pickering and Henderson (1966) reported 96-hr LC50 values of 4.85 (C.L. 3.55 - 6.32), 5.37 (C.L. = 3.84 - 7.00), 5.46 (C.L. = 4.29 - 6.94), and 5.82 mg/ℓ (C.L. = 4.61 - 7.48) in soft water at 25°C. Cairns and Scheier (1957a) reported values between 2.86 and 3.78 mg/ℓ in soft water at 18°C and 1.93 - 3.63 mg/ℓ at 30°C. The work of Cairns and Scheier (1957b) resulted in the highest reported 96-hr median lethal concentration (8.02 mg/ℓ) for these conditions. The 96-hr LC50 of 3.21 mg/ℓ (C.L. = 2.10 - 4.61) determined during this study is comparable to the values reported in the literature.

Copper

The acute toxicity of copper to bluegills under similar conditions has also been reported using copper sulfate or cupric chloride as a source of copper. Pickering and Henderson (1966) reported a 96-hr LC50 of 0.66 mg/ℓ (C. L. = 0.52 - 0.85) at 25°C. Trama (1954) reported a value of 0.74 mg/ℓ at 20°C; Patrick et al. (1968) reported a value of 1.25 mg/ℓ at 18°C; and Benoit (1975) reported a value of 1.1 mg/ℓ at 20°C. The value of 1.0 mg/ℓ (C.L. = 0.9 - 1.2) determined here compares favorably with those values.

Nickel

Few reports of the acute toxicity of nickel to bluegills under similar conditions are to be found in the literature. Pickering and Henderson (1966) used nickelous chloride and reported 96-hr LC50s of 5.18 mg/ℓ (C.L. = 2.02 - 6.88) and 5.36 mg/ℓ (C.L. = 4.4 - 6.74) at 25°C in water of 20 mg/ℓ total hardness. In water of 360 mg/ℓ, the same authors reported an LC50 of 39.6 mg/ℓ (C.L. = 33.7 - 45.3) for nickel.

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TABLE 3. RESULTS OF COMBINED TOXICITY TESTS

Toxicant		LC50 (mg/ℓ)	95 percent C. L.	Total fish
	Zn^{++}	3.2	2.1 - 4.6	120
	Cu^{++}	1.0	0.9 - 1.2	120
	Ni^{++}	21.2	18.1 - 25.5	120
	Cr^{+6}	132.9	113.6 - 155.7	117*
MixA	Zn^{++}	1.4	1.0 - 2.0	119†
MixA	Cu^{++}	0.4	0.3 - 0.6	
MixB	Zn^{++}	0.9	0.7 - 1.3	118*
MixB	Cu^{++}	0.5	0.2 - 1.2	
MixB	Ni^{++}	10.0	4.6 - 23.1	
MixB	Cr^{+6}	61.6	47.3 - 82.4	

*Three and two fish, respectively, died of behavioral causes and were disregarded.

†Only 119 fish at start due to counting error.

The LC50 of 21.2 mg/ℓ reported here falls between those two values. Our tests were carried out in water with a total hardness of 32 - 71 mg/ℓ, values that are closer to the soft water of Pickering and Henderson than to their hard water. Pickering and Henderson used static tests and reported only the calculated concentrations of Ni^{++} while the present tests were continuous flow and the Ni^{++} concentrations were actually measured five times during the course of the tests. It is also of interest that the measurements are for total nickel in acidified samples.

Chromium

The acute toxicity of chromium (potassium chromate) has also been reported in the literature. Trama and Benoit (1960) reported an LC50 of 170 mg/ℓ for hexavalent chromium in water with a total hardness of 45 mg/ℓ. The LC50 of 132.9 mg/ℓ (C.L. = 113.6 - 155.7) determined for hexavalent chromium during this study agrees fairly well with those results. Trama and Benoit used static toxicity tests and apparently reported calculated concentrations rather than measured concentrations for chromium. Again, it is well to remember that the concentrations given in the present report are measures of the total chromium present in the acidified sample.

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MixA

The acute toxicity of zinc-copper mixtures to bluegills has apparently not been reported in the literature.

An MAI of +0.218 was calculated with the data in Table 3. The calculated index range (-0.644 to +1.304) includes 0, and, thus, we conclude that the toxicity of zinc-copper mixtures to bluegills is additive under the test conditions.

MixB

The acute toxicity of zinc-copper-nickel-chromium mixtures to bluegills has not been reported in the literature.

MAI for this mixture is -0.738, which would seem to indicate a tendency toward antagonistic toxicity. However, the calculated index range of -2.982 to +0.193 indicates that this is not a significant departure from additive toxicity. It should be kept in mind that the actual toxicity ratio during these tests was 1:1.8:1.7:1.6, not the desired 1:1:1:1. Before discounting the possibility that these metals display reduced toxicity in mixture, additional toxicity tests should be carried out.

VENTILATORY STUDIES

All living organisms display considerable natural variability in morphological, physiological, and behavioral parameters. Variability in the ventilatory behavior of fish was both expected and found; this quality makes change and adaptability possible--and makes analysis of biological data difficult.

Strip chart figures illustrating variability in ventilatory rates as well as in the shape and amplitude of the signal are included in Appendix D. These figures, especially those representing interval 2 (the pretreatment interval), show that the analysis of short intermittent segments of such recordings make characterizing ventilatory behavior difficult. The figures in Appendix D can be placed in perspective by comparison with Figures B-5 and C-5 which indicate the average rates and amplitudes, respectively, for these fish. The strip chart recordings from all 4 fish were taken at the same points during run 5 (copper, pattern 2).

Diurnal Variability

It has been shown that natural diurnal light cycles have a definite effect on the ventilatory behavior of fishes (Morgan et al., 1979; van der Schalie, 1977). By exposing the fish to continuous 24-hr light, it was hoped that these effects would be removed to simplify analysis of data. The graphic data for the

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pretreatment intervals in Appendices B and C show no obviously identifiable cycles.

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Other Variability

It can also be seen from Appendices B, C, and D that a great deal of unexplained individual variability as well as variability between individuals was apparent. One example of variation that can be explained can be noted in run 1 (Figs. B-1, C-1). At about hour 48, a malfunction of the temperature controller caused the temperature to drop from 22°C to 16°C in less than 15 min and remain there for approximately 6 hr at which point the temperature was returned to 22°C at the same rate. A rather high peak for 4 or 5 hr is apparent for many of the fish. This corresponds to the return to 22°C and is the result of both increased rates and amplitudes. The rather quick return to prestress levels and the maintenance of those levels among the controls indicate that there was no deleterious effect on the fish, at least for the duration of the test.

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General Trends

A total of 55 control fish were monitored during this study. The average ventilatory rate for these fish during the pretreatment interval was 31.7 counts per minute (range, 15.9 - 51.0). During the treatment interval, the average rate was 31.0 counts per minute (15.9 - 45.8), and during the post-treatment interval, it was 28.7 counts per minute (14.2 - 43.8). The average rate for the other 125 fish during the pretreatment period was 28.5 counts per minute (14.3 - 55.4). It is possible that the small downward trend in rate is related to physical condition over time since the fish were not fed during the tests. Van der Schalie (1977) visually observed rates of 34.8 counts per minute (28.0 - 48.0) during daytime observation.

The average amplitude during pretreatment for the controls was 5.5 volts (2.5 - 9.9); during the treatment interval, it was 6.0 volts (1.8 - 12.3); and during post-treatment, 5.4 volts (1.8 - 14.2). The average pretreatment value for the treated fish was 5.8 volts (2.5 - 14.2). No obvious trends are apparent.

Changes in Rate

During the treatment interval, 26 control fish increased their average rate with respect to the pretreatment interval while 29 decreased. The average change was a decrease of only 0.7 counts per minute (2.2 percent). Average ventilatory rate for 115 fish that received a toxicant treatment increased during this interval while only 10 decreased. The average change here was an increase of 15.5 counts per minute (54.4 percent).

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During the post-treatment interval, 39 of the control fish decreased in average rate with respect to the treatment interval while 16 showed an increase. The average change was a decrease of 2.3 counts per minute (7.4 percent). Of the 125 treated fish, 123 survived the treatment interval, and 111 of these decreased in average rate during the post-treatment interval while 12 showed an increase. The average change for these fish was a decrease of 13.1 counts per minute. This was a 29.8 percent decrease with respect to the treatment interval but 46.0 percent with respect to the pretreatment interval. This can be compared with the 54.4 percent increase during the treatment interval.

The obvious conclusion is that sublethal, heavy metal stress causes increased ventilation rates in bluegills while the removal of that stress results in a return to rates near the pretreatment levels.

Changes in Amplitude

During the treatment interval, 27 of the control fish decreased in average amplitude with respect to the pretreatment interval and 28 increased. The average change was an increase of 0.4 volts (7.3 percent). Average amplitude decreased in 107 treated fish while 18 responded with an increase. The average change for these fish was a decrease of 2.1 volts (36.2 percent).

During the post-treatment interval, 21 of the control fish increased in average amplitude and 34 decreased. The average change was a decrease of 0.6 volts (10.0 percent) with respect to the treatment interval. Of the 123 surviving toxicant exposed fish, 112 increased their average amplitude during this interval while only 11 decreased. The average change for these fish was an increase of 2.4 volts. This was a 64.9 percent increase with respect to the treatment interval and a 41.4 percent increase with respect to the pretreatment interval. This value can be compared with the 36.2 percent decrease during the treatment interval.

It is apparent from this that the application of these toxicants caused a marked decrease in the average amplitude of the ventilatory signals. It is further apparent that recovery to near pretreatment levels is indicated with the cessation of toxicant application.

Toxicant Effects on the System

It is known that large increases in conductivity or, correspondingly, reductions in resistance can greatly reduce the amplitude of the ventilatory signal. Cairns et al. (1980) reported that 29 $^{o}/_{oo}$ sea water reduced the signal amplitude so much that a considerable increase in amplification was necessary in order to adapt this system to marine conditions. This raises a question with regard to the source of the reduction in signal amplitude

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observed when toxicant was applied. Since any addition of dissolved salts can reduce the electrical resistance of the water between the electrodes (see conductivity values, Table A-1), it is possible that the observed change in amplitude is due to the effect of the toxicant on the electrical resistance of the system and not to an effect on the fish.

It is possible to measure the total resistance of a ventilatory sensing chamber by measuring the resistance when the loading resistor is removed and a volt-ohm meter is attached in its place. The total resistance for a normally operating ventilatory sensing chamber is then the inverse of the inverse of the sum of the measured resistance and the 10,000Ω loading resistor. The total resistance was measured for a chamber filled with diluent water, various concentrations of the individual toxicants up to 120 mg/ℓ (dissolved in diluent), and diluent water with a fish in place. The fish itself had no measurable effect on the total resistance, but the dissolved salt content did. The values ranged from a high of 8389Ω with diluent or diluent and fish to a low of 7143Ω with a solution of 120 mg/ℓ Cu^{++}.

It was then possible to simulate the change in total system resistance that would occur with the addition of various concentrations of toxicant by changing the values of the loading resistor while only diluent was passed through the chamber. Preliminary data from an acclimated fish indicated that in order to reduce the signal amplitude by 24.8 percent it is necessary to reduce the total system resistance to approximately 6000Ω. The amplitude reduction indicated for the measured solutions (100 - 200 mg/ℓ) was 12-13 percent. The maximum concentration delivered during this study, test 12, would cause an amplitude reduction of less than 10 percent. Thus, the average amplitude reduction of 36.2 percent would be due primarily to the effects of the toxicant on the fish, not on the electronic system. It is also significant to note that only in tests 11 and 12 did the toxicant concentration reach levels that would depress the amplitude by more than 2 percent.

Distribution of Interval Means

The distribution of each of the 12 sets of interval means for the ventilatory rates was compared with the normal distribution using a chi squared goodness of fit test. The results of these analyses are presented in Table 4. The null hypothesis that no difference exists could be accepted at a traditional alpha level of 0.05 for nine of these distributions. Two of the remaining three had probabilities for acceptance of null greater than 0.01 while only one had a probability for acceptance of less than 0.01.

The distribution of each of the 12 sets of interval means for average amplitude was also compared with the normal distribu-

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TABLE 4. RESULTS OF CHI SQUARED TESTS FOR GOODNESS OF FIT TO THE NORMAL DISTRIBUTION

Toxicant	Data set	Interval		
		Pretreatment	Treatment	Post-treatment
Zinc	Rate	0.80 - 0.90*	0.20 - 0.30	0.70 - 0.80
Zinc	Amplitude	0.20 - 0.30	0.20 - 0.30	0.30 - 0.50
Copper	Rate	0.10 - 0.30	0.05 - 0.10	0.20 - 0.30
Copper	Amplitude	0.05 - 0.10	0.02 - 0.05	0.70 - 0.80
MixA	Rate	0.01 - 0.02	0.001- 0.01	0.10 - 0.20
MixA	Amplitude	0.05 - 0.10	0.001>	0.02 - 0.05
MixB	Rate	0.10 - 0.20	0.70 - 0.80	0.01 - 0.02
MixB	Amplitude	0.50 - 0.70	0.02 - 0.05	0.30 - 0.50

*Probability for obtaining a value this large or larger due to chance.

tion using the chi squared goodness of fit test (Table 4). The null hypothesis of no difference could be accepted at the 0.05 alpha level for 8 of these 12 data sets. Three of the remaining four sets had probabilities for acceptance of null that were greater than 0.02, and only one was less than 0.01 ($P<0.001$).

Homogeneity of Variances

Cochran's C statistic was calculated for the same data sets indicated above (Table 5). Only 1 of the 12 data sets for ventilatory rate exceeded the tabulated values at the 0.05 alpha level. This indicated that, at that level, the null hypothesis (that the cells have homogeneous variances) was acceptable for 11 of the 12 intervals tested.

The same calculations were carried out for the corresponding amplitude data sets. It was possible to accept the null hypothesis at the 0.05 alpha level for 8 of the 12.

Acceptability of ANOVA

Two-thirds (16) of the 24 data sets tested met both criteria (normal distribution and homogeneity of variances) for the acceptability of parametric analyses, and only 3 failed both. As this indicates only minor or random departure from normality, the one-way ANOVA with its relatively robust F test appears to be an appropriate test for the analysis of these data.

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TABLE 5. COCHRAN'S C STATISTIC

Toxicant	Data Set	Interval: Pre-treatment	Interval: Treatment	Interval: Post-treatment	Table Value*	df†
Zinc	Rate	0.457	0.492	0.298	0.5967	11/3
Zinc	Amplitude	0.694	0.627	0.286		
Copper	Rate	0.429	0.468	0.565	0.5867	12/3
Copper	Amplitude	0.356	0.511	0.497		
MixA	Rate	0.259	0.335	0.340	0.5667	13/3
MixA	Amplitude	0.514	0.588	0.506		
MixB	Rate	0.477	0.389	0.717	0.5667	13/3
MixB	Amplitude	0.503	0.429	0.648		

*Values that do not exceed the table value indicate homogeneous variances.

†Largest N-1/Number of variances - 1.

Results of ANOVA

The results of the one-way analysis of variance tests are presented in Tables 6 and 7. In Table 6, the smaller the probability (P) values are the greater the chance that there is a detected significant difference among the ventilatory rates or the average amplitudes with respect to the patterns of application of toxicant.

The Duncan's Multiple Range test was used in an attempt to better define the indicated differences. In Table 7, the detected differences are set off by commas. However, if one pattern appears in two or more different groups within a single cell in this table, these differences are blurred. The most significant differences here are for patterns that are set apart from all other patterns. For example, during the treatment interval for the average ventilatory rates for MixB (Table 7), Duncan's Multiple Range test indicates that the controls are significantly different from all other patterns and that no difference can be detected between patterns 1, 2, and 3, a conclusion that can be confirmed from Figures B-10, B-11, and B-12.

It can be seen from the F values in Table 6 that, with one exception, there is no significant difference indicated between ventilatory rates for any of the four patterns at the traditonal alpha level of 0.05 during either the pretreatment or the post-treatment time intervals. With only two exceptions, this holds

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TABLE 6. RESULTS OF ONE-WAY ANOVA.

		Interval								
		Pretreatment			Treatment			Post-treatment		
Toxicant	Data Set	F	P<F*	df	F	P<F	df	F	P<F	df
Zinc	Rate	2.77	0.0550	3,37	4.05	0.0138	3,37	2.50	0.0743	3,37
Zinc	Amplitude	7.57	0.0005	3,37	8.62	0.0002	3,37	1.58	0.2109	3,37
Copper	Rate	1.26	0.3004	3,39	3.16	0.0351	3,39	2.29	0.0934	3,38
Copper	Amplitude	0.50	0.6839	3,39	10.28	0.0001	3,39	2.71	0.0589	3,38
MixA	Rate	1.54	0.2166	3,44	3.35	0.0274	3,44	1.74	0.1733	3,44
MixA	Amplitude	1.53	0.2206	3,44	10.64	0.0001	3,44	1.01	0.3992	3,44
MixB	Rate	3.58	0.0211	3,44	12.25	0.0001	3,44	1.72	0.1780	3,43
MixB	Amplitude	3.07	0.0373	3,44	6.79	0.0007	3,44	2.48	0.0735	3,43

*All P values less than 0.05 could be considered significantly different at the 95% level.

TABLE 7. RESULTS OF DUNCAN'S MULTIPLE RANGE TEST AT THE 95 PERCENT LEVEL OF SIGNIFICANCE.

Toxicant	Data Set	Interval: Pretreatment	Interval: Treatment	Interval: Post-treatment
Zinc	Rate	0 = 1 = 3, 0 = 2*	0, 1 = 2 = 3†	0 = 3, 1 = 2 = 3
Zinc	Amplitude	0 = 1 = 3, 3 = 1, 2	0 = 3, 3 = 2, 1	0 = 1 = 2 = 3
Copper	Rate	0 = 1 = 2 = 3†	0 = 3, 1 = 2 = 3	0 = 1, 0 = 3 = 2
Copper	Amplitude	0 = 1 = 2 = 3	0, 1 = 2 = 3	0 = 2 - 3, 0 = 1 = 2
MixA	Rate	0 = 1 = 2 = 3	0 = 3, 3 = 2 = 1	0 = 1 = 2 = 3
MixA	Amplitude	0 = 1 = 2 = 3	0 = 3, 2 = 1	0 = 1 = 2 = 3
MixB	Rate	0 = 3, 3 = 1 = 2	0, 1 = 2 = 3	0 = 1 = 2 = 3
MixB	Amplitude	0 = 1 = 2, 3	0, 1 = 2 = 3	0 = 1 = 2, 1 = 2 = 3

*0 = pattern 0 (control); 1 = pattern 1; 2 = pattern 2; 3 = pattern 3.

†Significantly different patterns are set off by commas; however, if one pattern appears in two or more different groups within a single cell, the differences are less sharp. For example 0, 1=2=3 indicates that pattern 0 is significantly different from all other patterns while patterns 1, 2, and 3 cannot be statistically differentiated. 0=1=2=3 indicates no differences are detectable.

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true for the average amplitudes as well.

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It is significant to note that in all instances the probabilities that no differences exist are much smaller (in most cases an order of magnitude) during the treatment intervals than during the corresponding pre- and post-treatment intervals. The indication being that the significant differences are much greater during the treatment period, again a conclusion that can be verified from the graphed data Appendices B and C.

The results of the Duncan's Multiple Range test (Table 7) reveal no clear cut pattern during pretreatment and post-treatment periods. While differences are indicated for 8 of the 16 data sets, only 2 of these set a single pattern off from all others, and these are different patterns in each case.

During the treatment interval, however, differences are indicated for all eight data sets with pattern 0 (controls being differentiated from all others most often (four times). When 0 was not differentiated by itself, it was always (four times) included in a group with pattern 3, and in three of these cases pattern 3 was also included in other sets.

It can be concluded that while these results supply no absolutes, there is a definite trend that indicates statistically that the effects of sublethal doses of these toxicants are definitely detectable using either average ventilatory rates or average ventilatory amplitudes or preferably both.

The various application patterns chosen for this study cannot be differentiated as easily. When toxicant was applied in pattern 1, apparent acclimation occurred, especially during run 4 (Fig. B-4, C-4). However, when the toxicant was applied in intermittent bursts, the reaction to subsequent exposures was often as strong as it was to the first exposure. These differences have been statistically obscured by the extreme smoothing of data (calculation of interval means) necessary for these analyses. Appropriate methods do exist for the analysis of the ventilatory data for an individual fish (Box and Tiao, 1975) but are extremely complicated and time consuming and are, at present, not practical for quick analysis of large data sets such as these.

Multiple Regression

The forward stepwise, multiple regression indicates little or no predictability for any of the five variables used in the analysis: pattern, standard length, average toxicity ratio, total dose, or maximum concentration. In all cases, less than 25 percent of the variability in ventilatory rate was accounted for by these independent variables. None of the one variable models for all toxicants yielded a significant F ratio. Furthermore, only

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application pattern in the copper analysis had a significant F ratio in any of the multiple regression models (two). However, this was of no real importance as the overall F ratios for these two models were not significant.

It appears, from examination of the figures in Appendices B and C, that the fish responded more sharply to the higher concentrations of toxicant encountered in patterns 2 and 3 than to the lower concentrations encountered during pattern 1. It is possible that the failure of the statistical analyses to detect differences due to either application pattern or the maximum toxicant concentration was due to the extreme smoothing of the data before it was analyzed.

Possible Causes

Some workers have indicated that the mechanism by which heavy metals cause death is related to reduced diffusion capacity of the gills with resulting lower oxygen levels in the blood (Skidmore, 1970; Burton et al., 1972). It would seem reasonable to hypothesize that such hypoxia might be the cause of the increased ventilatory rates seen during this study. Others have reported increased ventilatory rates with hypoxia (Skidmore, 1964; Gee et al., 1977). When the water supply was halted to one of the sensing chambers, the dissolved oxygen level quickly fell (3 hours) to less than 3.5 mg/ℓ. The ventilatory rate increased from 45 counts per minute to 130-140 counts per minute; however, the amplitude also increased nearly 300 percent, the opposite of the demonstrated amplitude reaction to sublethal levels of heavy metals. While this is only preliminary data, it seems to indicate some other physiological reason for the increased ventilatory rates.

The studies indicated above were all carried out with very concentrated solutions, concentrated enough to cause rapid death, and emphasized gill damage and hypoxia (Skidmore, 1970; Burton et al., 1972). Most chronic studies have emphasized very low concentrations and have measured parameters such as growth, reproductive success, and various physiological responses (Christensen et al., 1972; Brungs et al., 1973; Benoit, 1975). None of these can presently be correlated with the short term, sublethal ventilatory effects described here. Perhaps an investigation of the osmoregulatory changes indicated by Lewis and Lewis (1971) and Katz (1979) might give a better insight into the physiological mechanisms involved.

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APPENDIX A

RESULTS OF WATER ANALYSES AND INDIVIDUAL TOXICITY TESTS

TABLE A-1. RESULTS OF CHEMICAL ANALYSES GIVEN AS THE AVERAGE OF THE FIVE ANALYSES FOR EACH FISH BIOASSAY CHAMBER

Test number	Tank number	Conductivity micromhos/cm	pH	Alkalinity (mg/l)	Total hardness (mg/l $CaCO_3$)
1 (Zinc)	1	110	7.5	26.8	22.4
	2V	123	7.4	28.0	28.8
	3IV	131	7.4	28.4	32.8
	4III	150	7.3	29.2	39.6
	5II	179	7.3	29.2	48.0
	I	204	7.2	29.2	59.2
2 (Zinc)	1	109	7.2	31.6	23.6
	2V	115	7.2	32.0	27.2
	3IV	118	7.1	31.2	28.4
	4III	121	7.0	30.0	31.2
	5II	129	7.0	30.4	35.6
	I	139	6.9	30.4	45.6
3 (Copper)	1	106	7.1	23.2	28.8
	2V	106	7.1	24.0	28.8
	3IV	106	7.1	24.0	28.8
	4III	106	7.0	24.0	28.4
	5II	106	6.9	24.0	28.0
	I	106	6.9	24.0	28.8
4 (Copper)	1	110	7.1	28.0	22.0
	2V	111	7.1	31.2	21.2
	3IV	112	7.1	30.4	23.3
	4III	113	7.1	30.4	23.6
	5II	115	6.9	30.8	24.4
	I	117	6.9	30.8	24.8
5 (Nickel)	1	118	7.1	30.0	36.6
	2V	128	7.1	32.8	34.6
	3IV	134	7.1	33.2	39.0
	4III	147	7.1	33.2	32.2
	5II	162	7.1	32.8	31.8
	I	194	7.1	32.8	33.2

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TABLE A-1 (cont.)

Test number	Tank number	Conductivity micromhos/cm	pH	Alkalinity (mg/l)	Total hardness (mg/l $CaCO_3$)
6	1	125	7.1	36.0	71.4
(Nickel)	2V	149	7.1	35.2	76.0
	3IV	163	7.1	35.6	65.2
	4III	189	7.1	36.0	67.6
	5II	225	7.1	35.8	68.4
	I	320	7.0	35.8	70.8
7	1	117	7.1	30.4	22.0
(Chromium)	2V	565	7.6	97.2	19.6
	3IV	876	7.8	139.6	22.4
	4III	1496	7.9	221.2	20.8
	5II	2052	8.2	361.6	20.8
	I	3352	8.3	551.0	20.8
8	1	111	7.1	40.8	44.4
(Chromium)	2V	256	7.2	63.6	43.6
	3IV	385	7.4	83.8	42.4
	4III	628	7.5	110.8	42.8
	5II	946	7.6	180.4	42.4
	I	1198	7.8	268.0	42.0
9	1	111	7.0	30.4	21.6
(MixA)	2V	114	7.0	30.0	21.2
	3IV	114	7.0	30.0	21.4
	4III	117	7.0	30.0	22.2
	5II	120	6.9	30.0	24.6
	I	126	6.8	30.0	26.6
10	1	118	7.1	30.4	29.2
(MixA)	2V	123	7.1	30.8	32.4
	3IV	125	7.1	31.6	32.8
	4III	127	7.1	32.8	32.8
	5II	130	7.0	32.4	34.8
	I	136	6.9	32.8	34.8
11	1	131	6.8	31.6	44.8
(MixB)	2V	263	7.1	53.8	40.4
	3IV	332	7.2	65.0	38.8
	4III	506	7.4	94.8	41.6
	5II	732	7.5	131.6	43.6
	I	1106	7.7	218.0	44.6

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TABLE A-1 (cont.)

Test number	Tank number	Conductivity micromhos/cm	pH	Alkalinity (mg/l)	Total hardness (mg/l $CaCO_3$)
12 (MixB)	1	119	7.1	33.0	35.0
	2V	310	7.3	57.2	43.0
	3IV	421	7.4	75.2	41.0
	4III	668	7.5	114.0	37.6
	5II	1106	7.6	171.6	38.6
	I	1719	7.6	269.2	35.6

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TABLE A-2. TOXICANT CONCENTRATIONS AND SURVIVAL DURING TOXICITY TESTS

Test number	Tank number	Average concentration (mg/l) Zn^{++}	Cu^{++}	Ni^{++}	Cr^{+6}	# Surviving at 96 hr (of 10)
1 (Zinc)	1 (Control)	0.0	-	-	-	10
	2V	2.6	-	-	-	9
	3IV	3.6	-	-	-	5
	4III	6.2	-	-	-	0
	5II	10.9	-	-	-	0
	I	23.2	-	-	-	0
2 (Zinc)	1 (Control)	0.1	-	-	-	10
	2V	1.2	-	-	-	7
	3IV	2.1	-	-	-	7
	4III	3.5	-	-	-	8
	5II	5.7	-	-	-	1
	I	9.9	-	-	-	0
3 (Copper)	1 (Control)	-	0.0	-	-	10
	2V	-	0.3	-	-	10
	3IV	-	0.5	-	-	10
	4III	-	0.8	-	-	10
	5II	-	1.4	-	-	0
	I	-	2.4	-	-	0
4 (Copper)	1 (Control)	-	0.0	-	-	10
	2V	-	0.5	-	-	9
	3IV	-	0.7	-	-	7
	4III	-	1.3	-	-	3
	5II	-	2.3	-	-	0
	I	-	4.0	-	-	0
5 (Nickel)	1 (Control)	-	-	0.0	-	10
	2V	-	-	2.3	-	10
	3IV	-	-	3.3	-	10
	4III	-	-	7.7	-	10
	5II	-	-	12.0	-	9
	I	-	-	20.9	-	6
6 (Nickel)	1 (Control)	-	-	0.1	-	10
	2V	-	-	7.1	-	10
	3IV	-	-	10.0	-	10
	4III	-	-	20.3	-	4
	5II	-	-	28.4	-	3
	I	-	-	50.0	-	0

TABLE A-2 (cont.)

Test number	Tank number	Average concentration (mg/l) Zn^{++}	Cu^{++}	Ni^{++}	Cr^{+6}	# Surviving at 96 hr (of 10)
7	1 (Control)	-	-	-	0.1	8*
(Chromium)	2V	-	-	-	37.6	9*
	3IV	-	-	-	62.8	10
	4III	-	-	-	119.8	8
	5II	-	-	-	181.0	3
	I	-	-	-	289.0	0
8	1 (Control)	-	-	-	0.1	10
(Chromium)	2V	-	-	-	66.8	8
	3IV	-	-	-	109.6	8
	4III	-	-	-	199.8	0
	5II	-	-	-	331.2	0
	I	-	-	-	525.2	-
9	1 (Control)	0.0	0.0	-	-	10
(MixA)	2V	0.4	0.1	-	-	10
	3IV	0.6	0.2	-	-	10
	4III	1.2	0.3	-	-	5
	5II	1.8	0.5	-	-	0
	I	3.1	0.9	-	-	0
10	1 (Control)	0.0	0.0	-	-	10
(MixA)	2V	0.4	0.1	-	-	10
	3IV	0.6	0.2	-	-	9
	4III	1.2	0.4	-	-	6†
	5II	2.0	0.5	-	-	8
	I	3.7	1.0	-	-	0
11	1 (Control)	0.0	0.0	0.0	0.0	10
(MixB)	2V	0.4	0.1	3.0	29.3	8
	3IV	0.6	0.2	4.8	36.6	10
	4III	1.0	0.5	8.9	72.4	7
	5II	1.9	0.8	14.6	99.6	0
	I	3.0	1.4	24.3	181.8	0
12	1 (Control)	0.0	0.0	0.0	0.0	8*
(MixB)	2V	0.4	0.3	5.9	30.2	9
	3IV	0.7	0.5	10.3	49.9	9
	4III	1.3	0.8	16.9	78.1	1
	5II	2.0	1.6	31.6	135.1	0
	I	3.1	2.6	46.7	226.0	0

*Two, one, and two disregarded due to behavioral death

†Only nine fish at the start, counting error

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APPENDIX B

Graphic representation of the average ventilatory rates for all 12 ventilatory tests. See text Figures 12, 13, and 14 and text table 1 for further details.

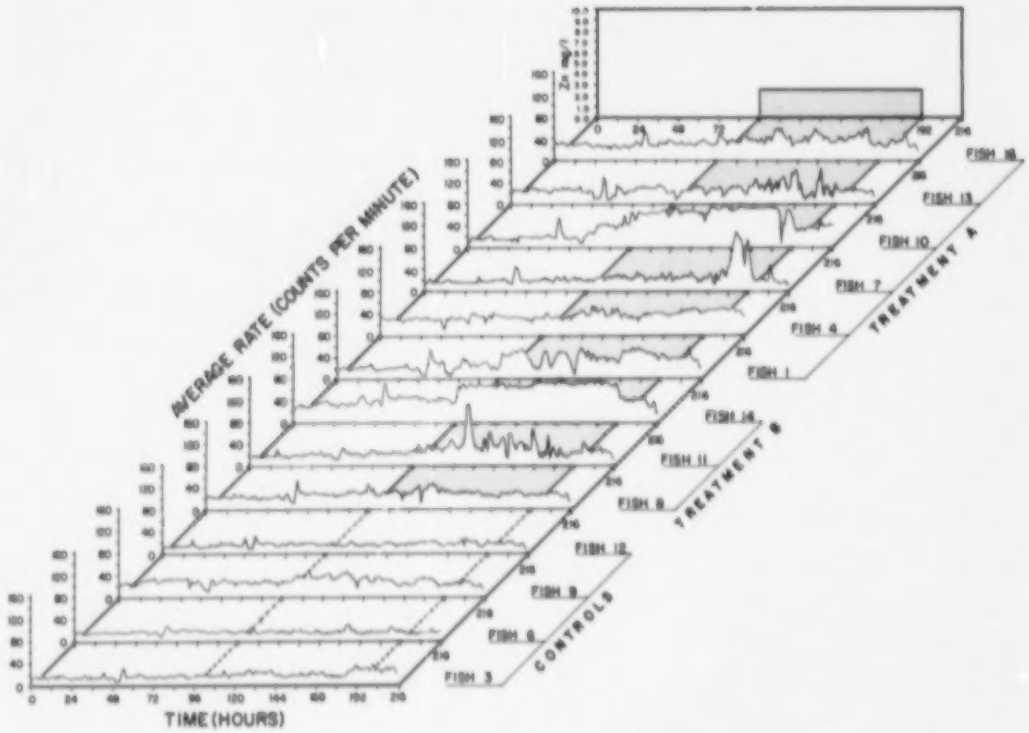

Figure B-1. Average ventilatory rates for ventilatory test 1. Toxicant = Zn^{++}, pattern = 1, concentration = 2.5 mg/l.

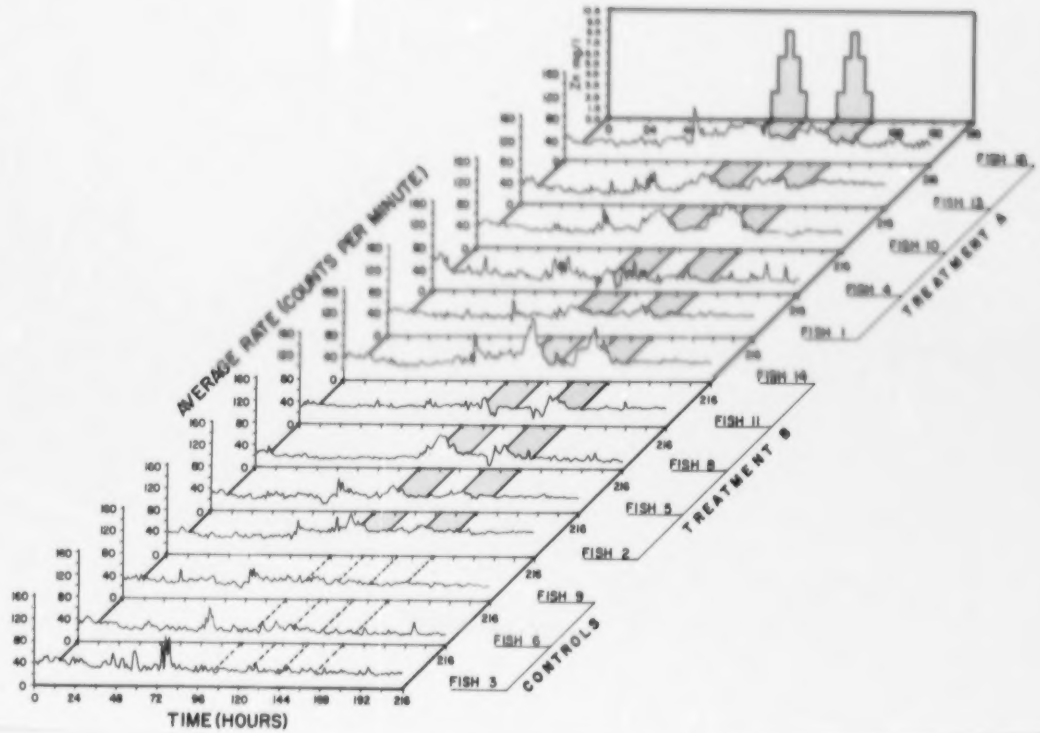

Figure B-2. Average ventilatory rates for ventilatory test 2. Toxicant = Zn^{++}, pattern = 2, concentrations = 2.5, 5.7, 8.1 mg/l.

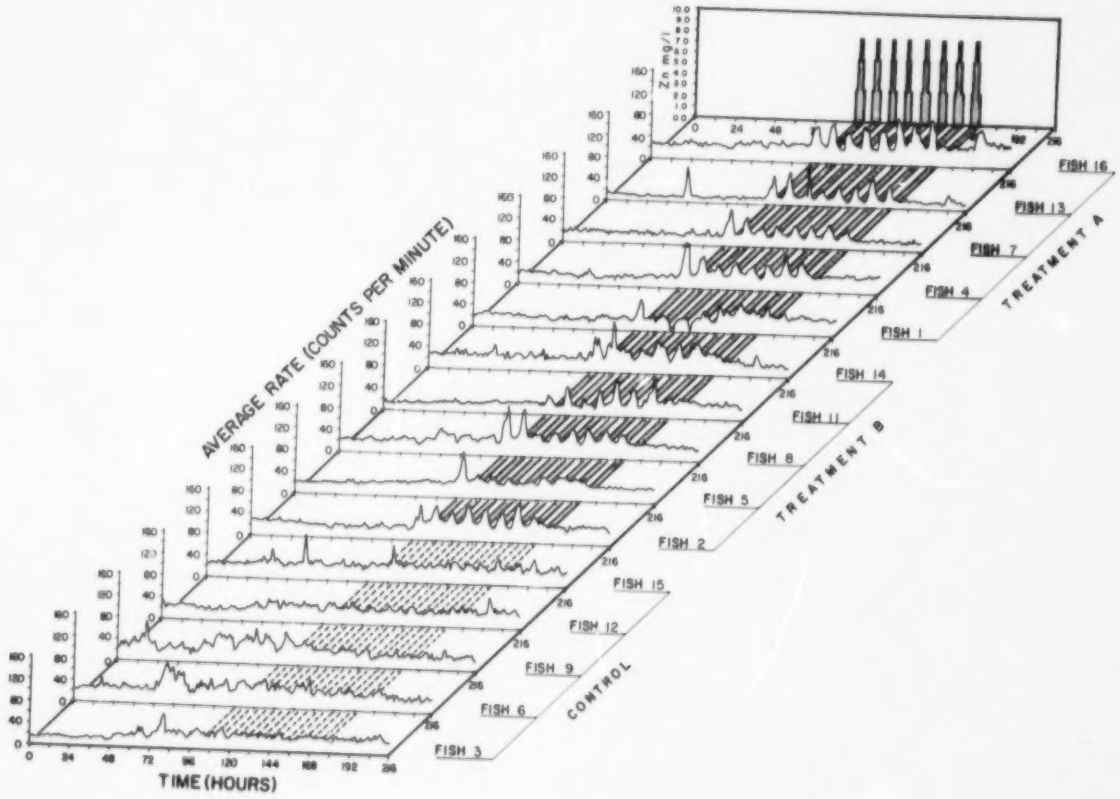

Figure B-3. Average ventilatory rates for ventilatory test 3. Toxicant = Zn^{++}, pattern = 3, concentrations = 2.9, 5.7, 7.7 mg/l.

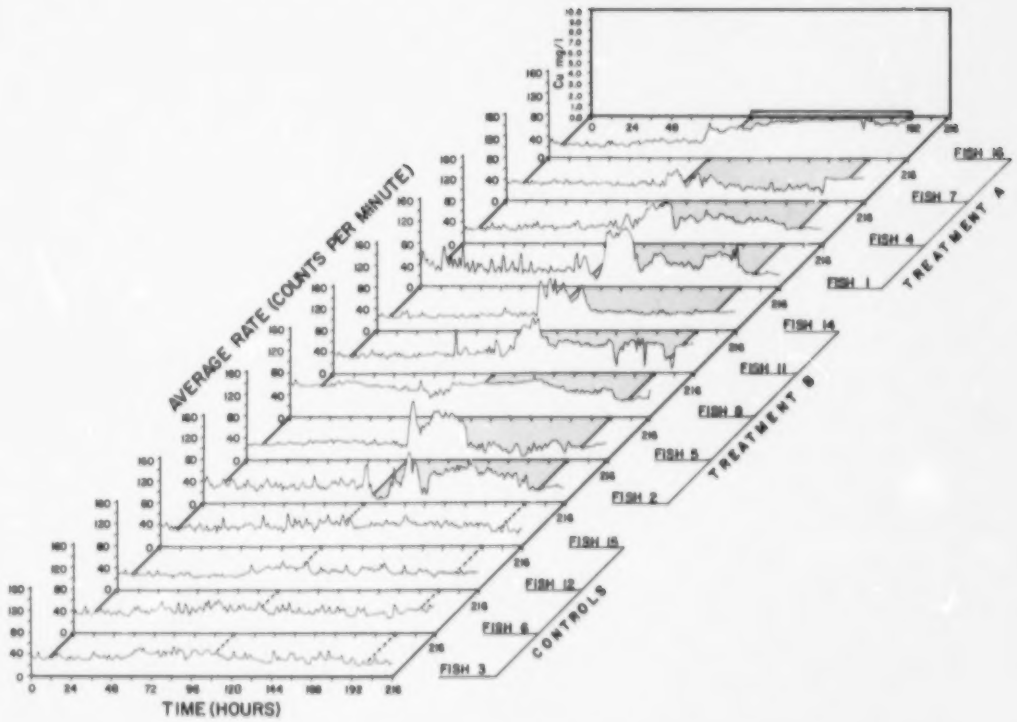

Figure B-4. Average ventilatory rates for ventilatory test 4. Toxicant = Cu^{++}, pattern = 1, concentration = 0.5 mg/l.

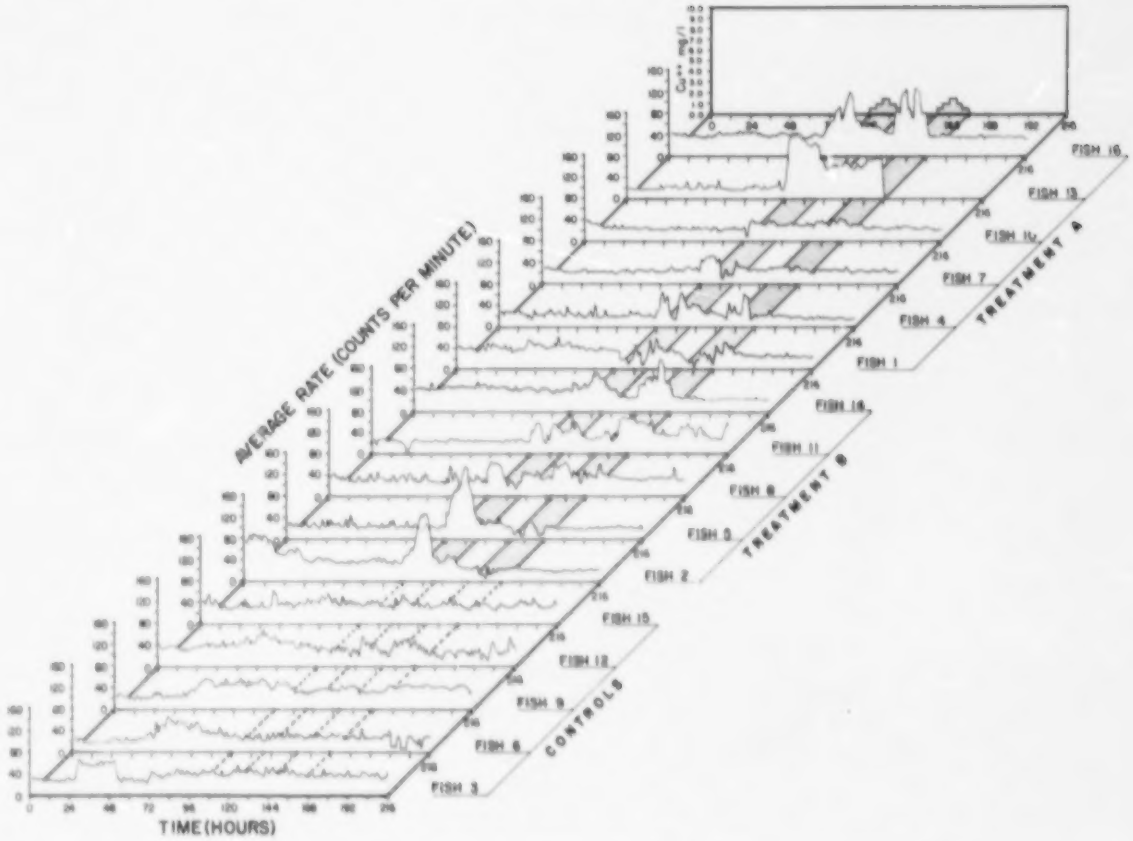

Figure B-5. Average ventilatory rates for ventilatory test 5. Toxicant = Cu^{++}, pattern = 2, concentrations = 0.5, 1.0, 1.5 mg/l. Representative strip chart records for number 5, 6, 7, and 8 can be found in Appendix D.

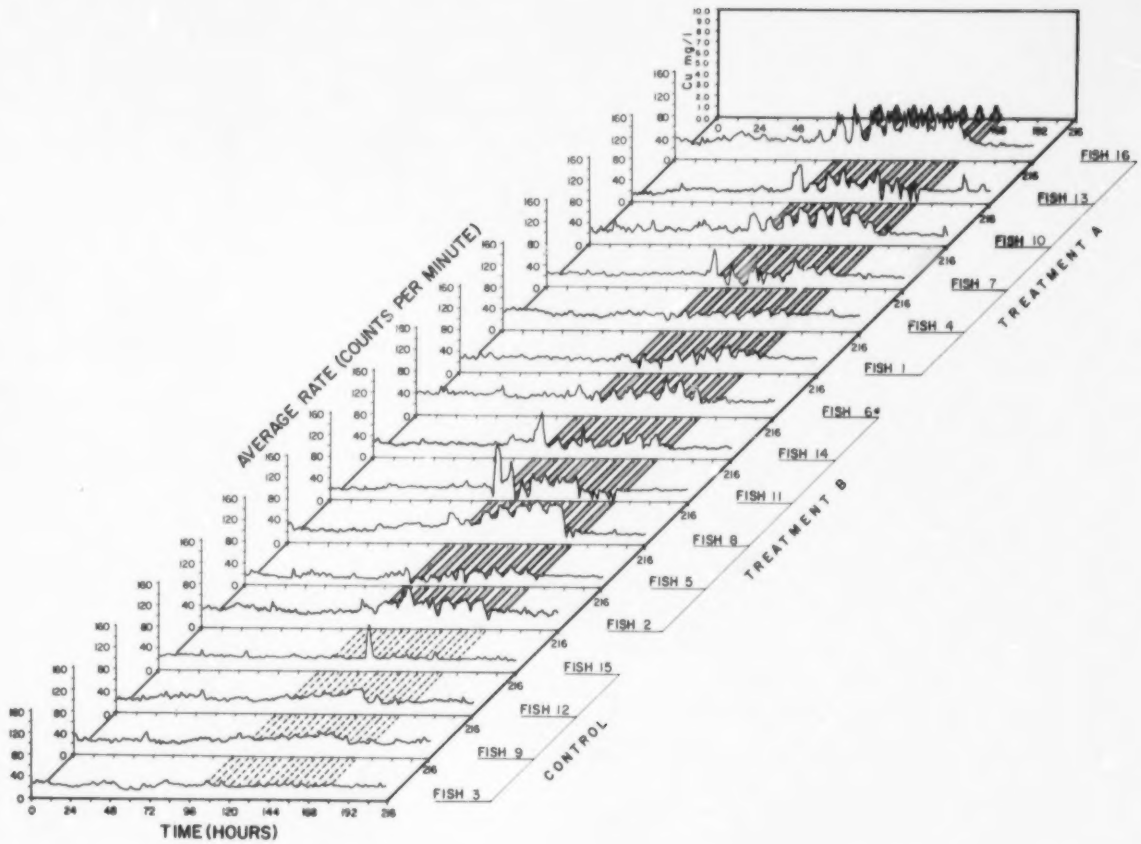

Figure B-6. Average ventilatory rates for ventilatory test 6. Toxicant = Cu^{++}, pattern = 3, concentrations = 0.5, 1.1, 1.3 mg/l. *Fish 6, a control, received a partial dose of toxicant due to splash and was not included in the statistical analysis.

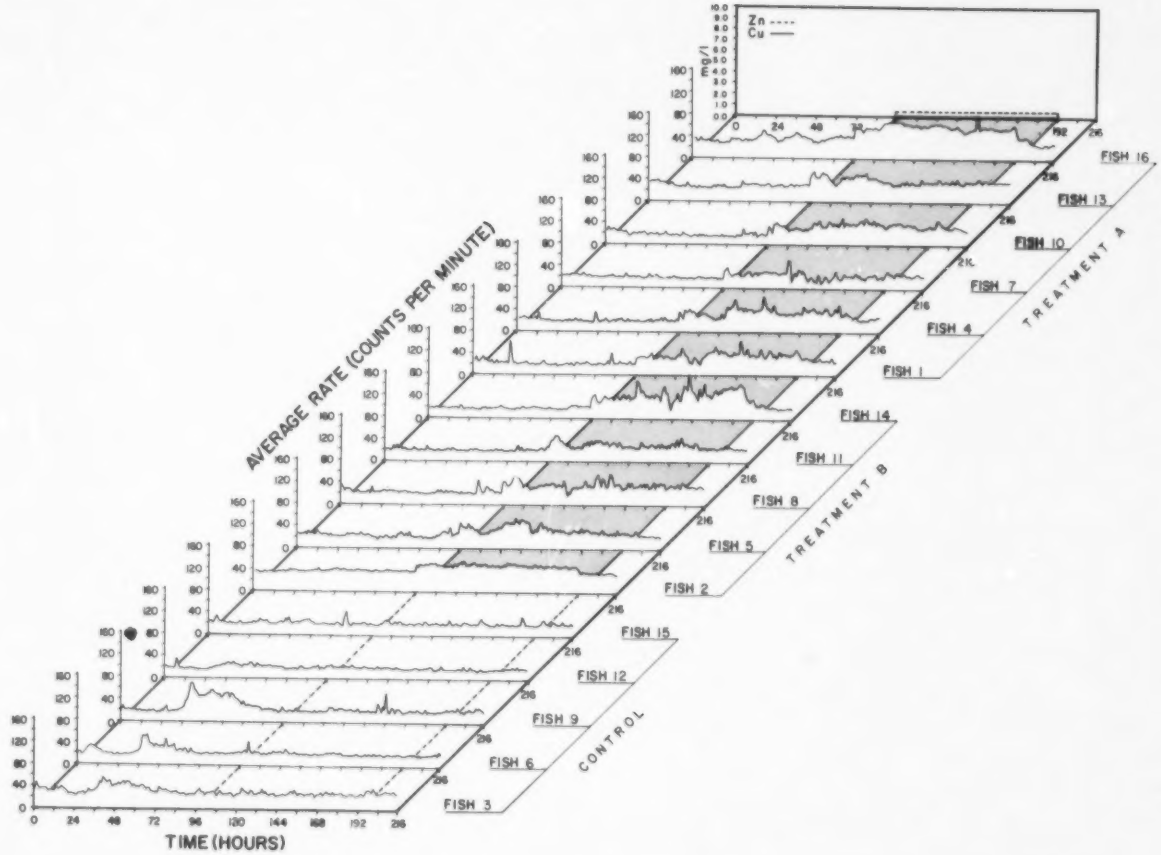

Figure B-7. Average ventilatory rates for ventilatory test 7. Toxicant = MixA, pattern = 1, concentrations = 0.6 mg/l Zn^{++}; 0.2 mg/l Cu^{++}.

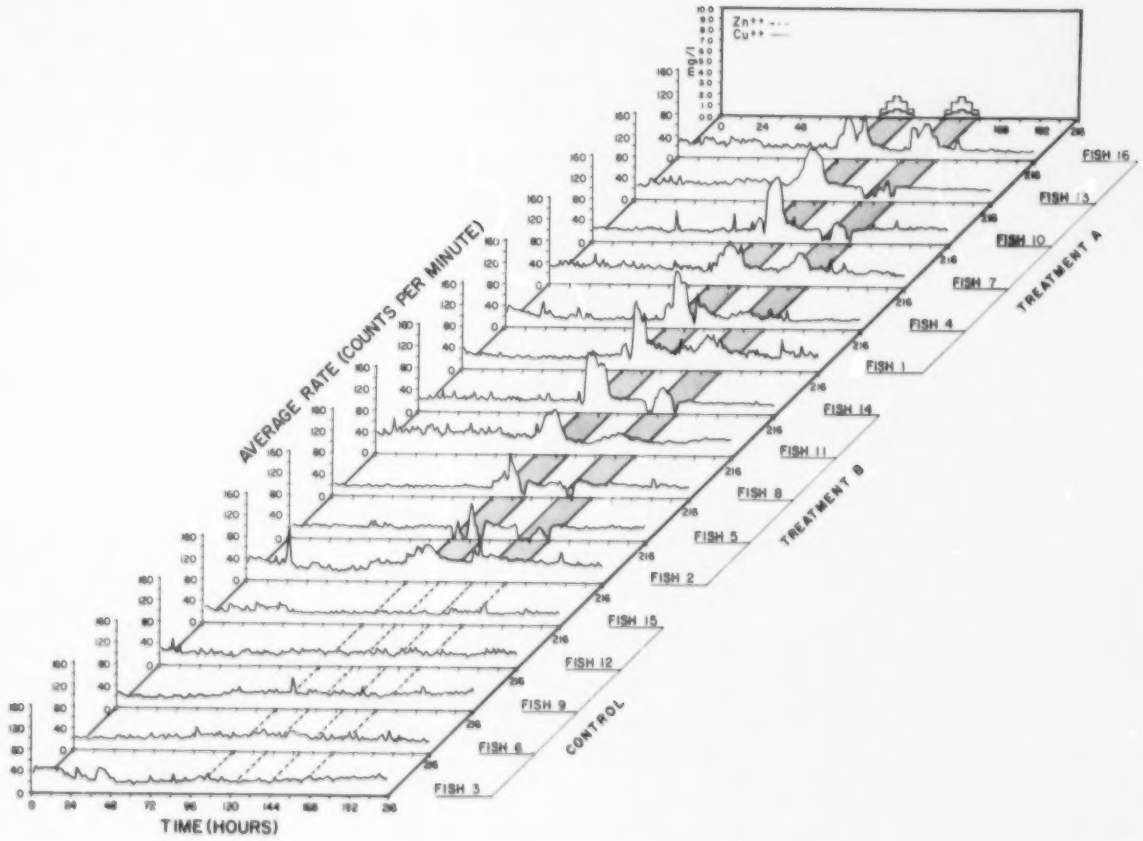

Figure B-8. Average ventilatory rates for ventilatory test 8. Toxicant = MixA, pattern = 2, concentrations = 0.6, 1.3, 1.8 mg/l Zn^{++}; 0.2, 0.4, 0.6 mg/l Cu^{++}.

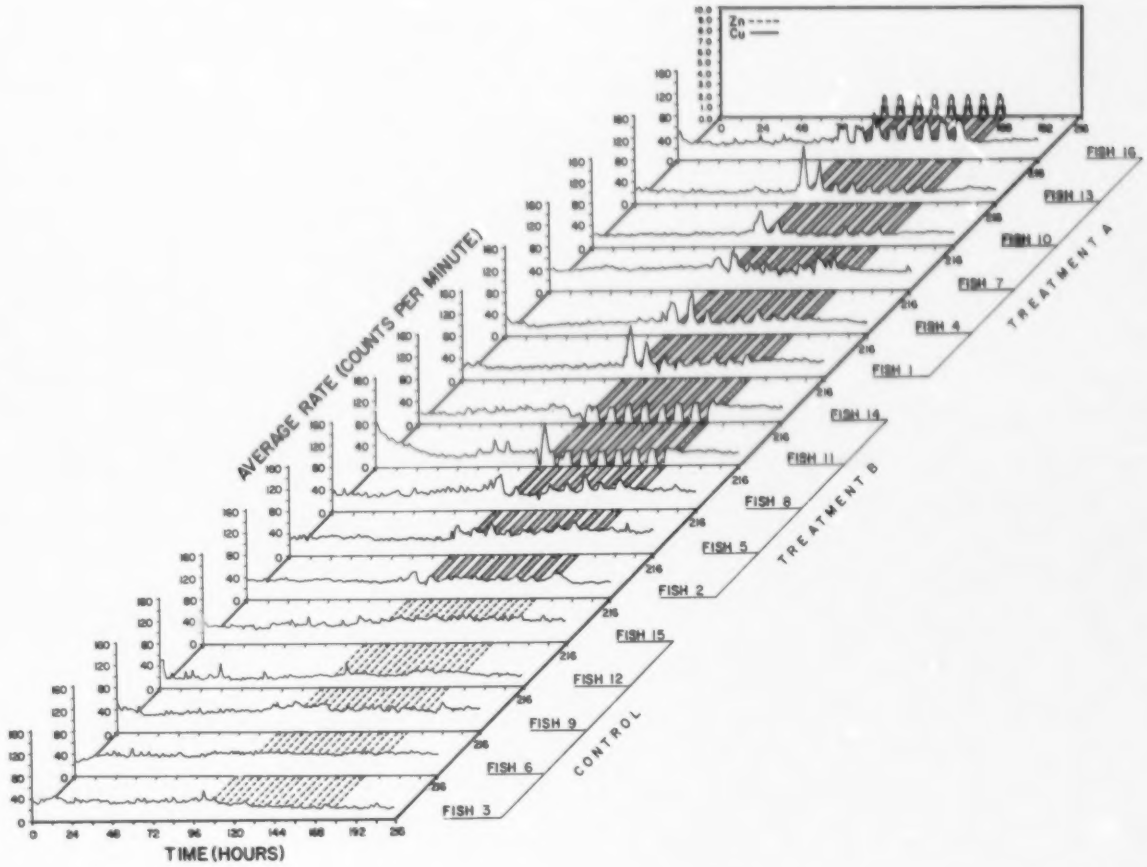

Figure B-9. Average ventilatory rates for ventilatory test 9. Toxicant = MixA, pattern = 3, concentrations = 0.6, 1.4, 1.8 mg/l Zn^{++}; 0.2, 0.5, 0.6 mg/l Cu^{++}.

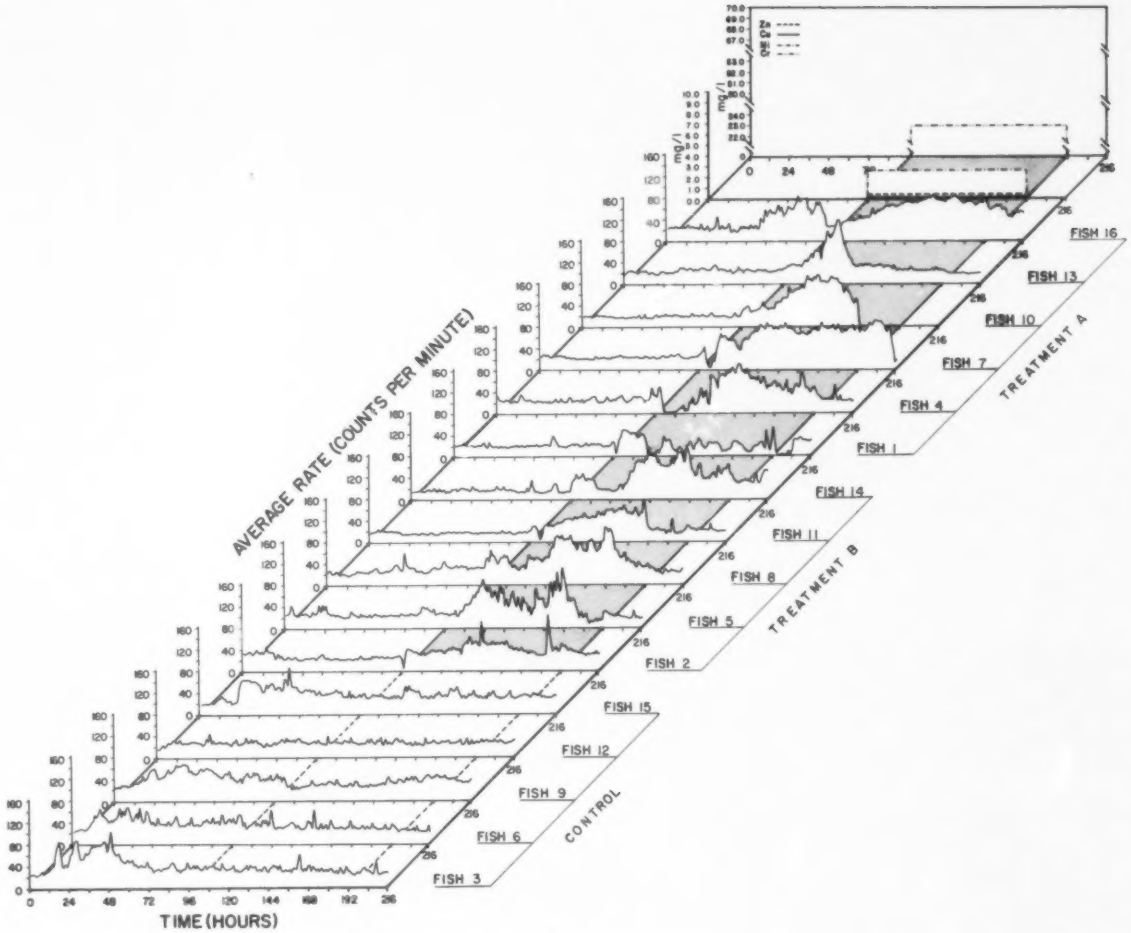

Figure B-10. Average ventilatory rates for ventilatory test 10. Toxicant = MixB, pattern = 1, concentrations = 0.3 mg/l Zn^{++}; 0.1 mg/l Cu^{++}; 2.5 mg/l Ni^{++}; 25.5 mg/l Cr^{+6}.

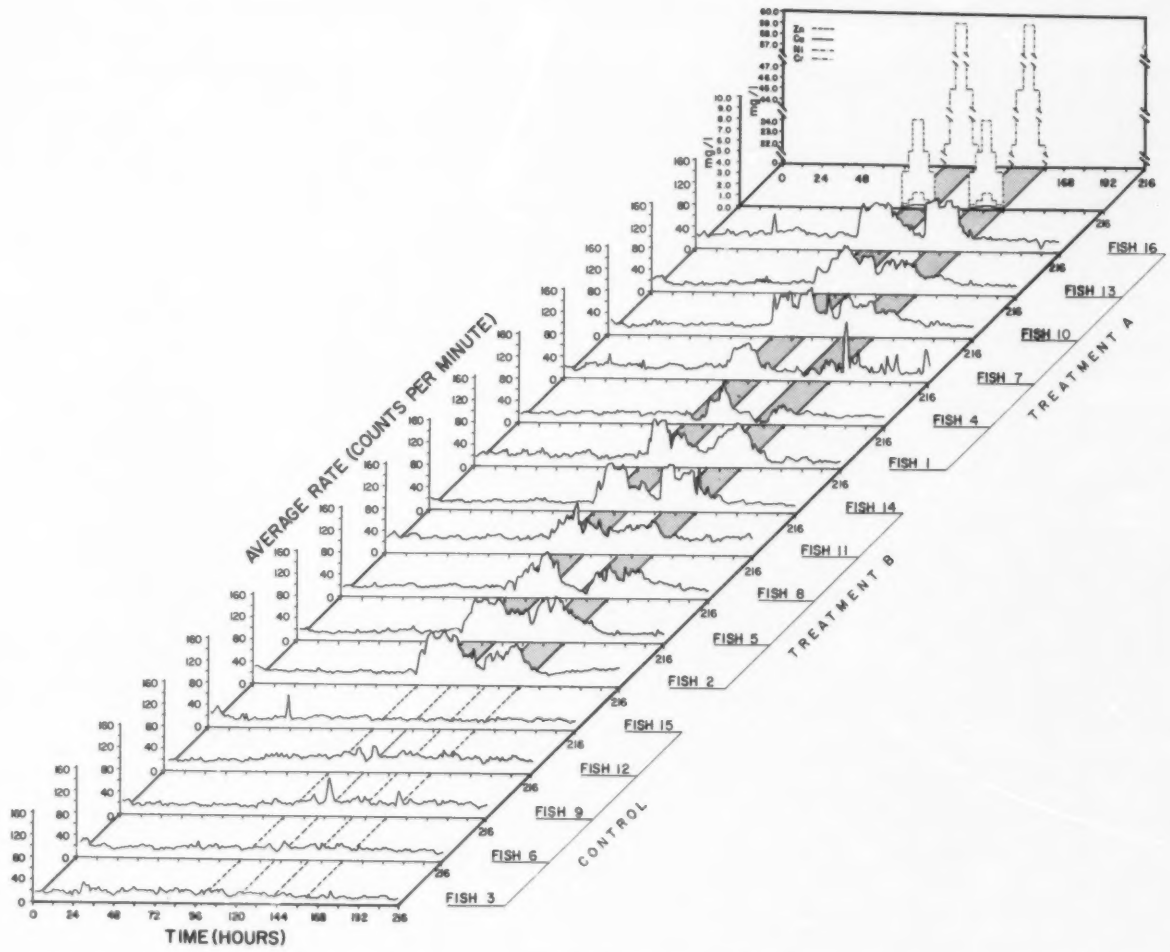

Figure B-11. Average ventilatory rates for ventilatory test 11. Toxicant = MixB, pattern = 2, concentrations = 0.4, 0.8, 1.1 mg/l Zn^{++}; 0.1, 0.2, 0.2+ mg/l Cu^{++}; 3.3, 5.1, 8.0 mg/l Ni^{++}; 22.5, 44.9, 58.7 mg/l Cr^{+6}.

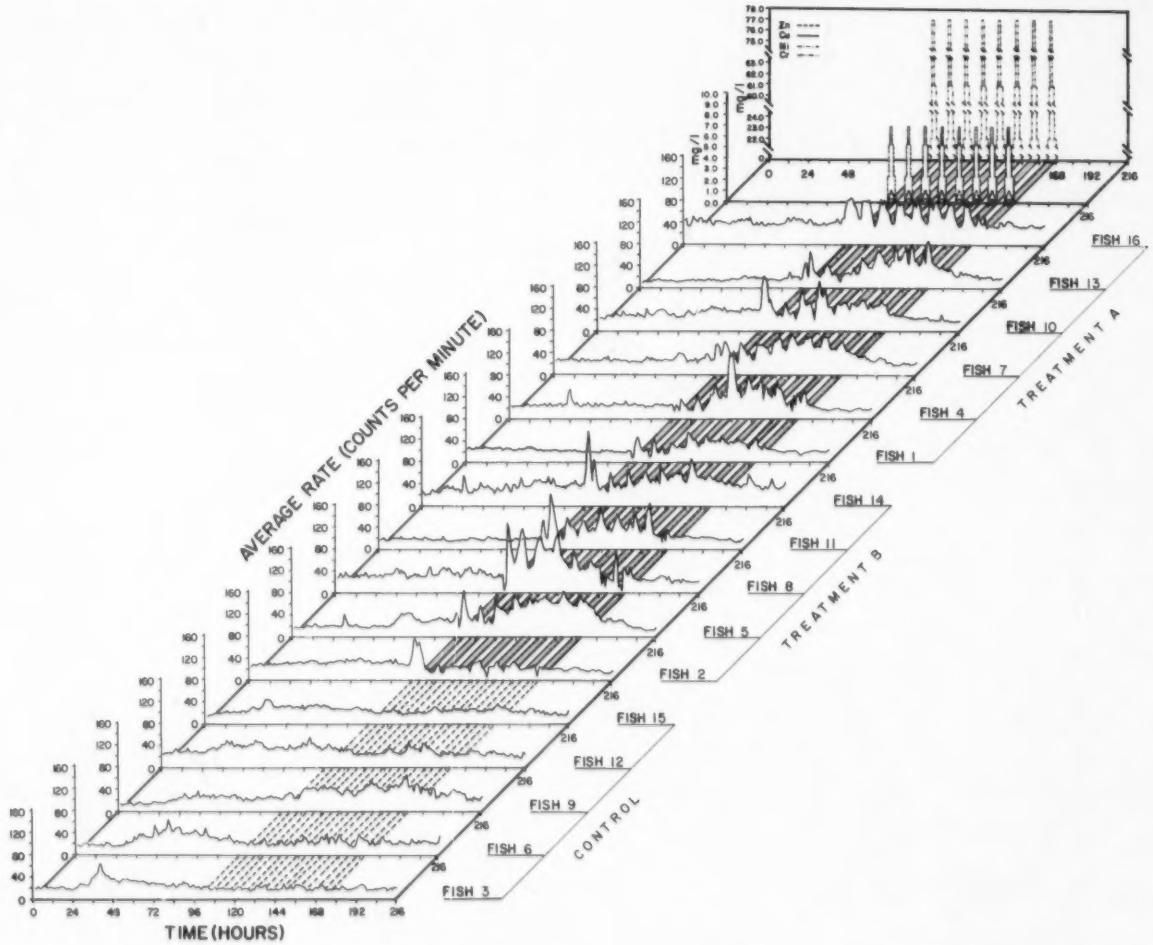

Figure B-12. Average ventilatory rates for ventilatory test 12. Toxicant = MixB, pattern = 3, concentrations = 0.4, 0.8, 1.1 mg/l Zn^{++}; 0.1, 0.2, 0.2+ mg/l Cu^{++}; 2.3, 5.3, 6.9 mg/l Ni^{++}; 22.3, 60.6, 77.0 mg/l Cr^{+6}.

APPENDIX C

Graphic representation of the average signal amplitude for all 12 ventilatory tests. See text Figures 12, 13, and 14 and text table 1 for further details.

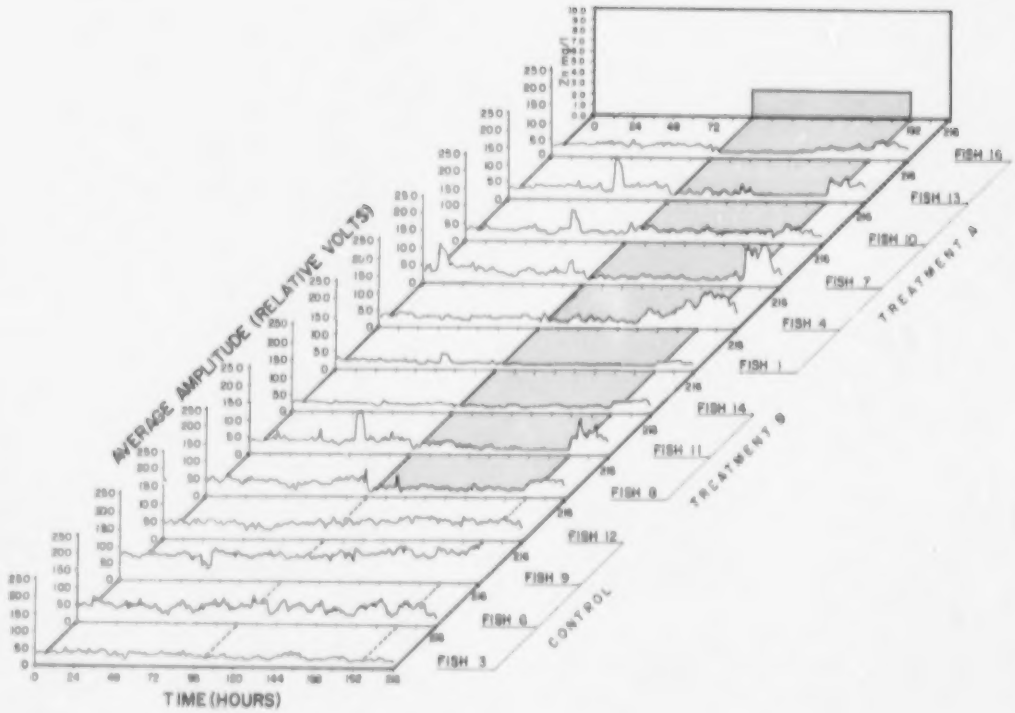

Figure C-1. Average ventilatory signal amplitudes for ventilatory test 1. Toxicant = Zn^{++}, pattern = 1.

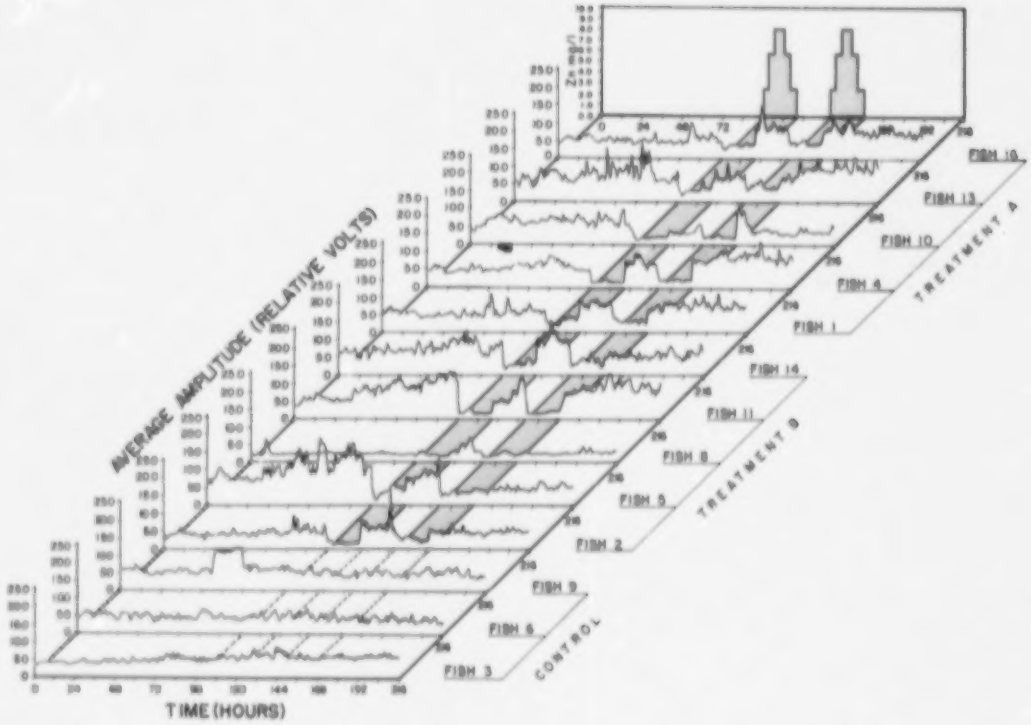

Figure C-2. Average ventilatory signal amplitudes for ventilatory test 2. Toxicant = Zn^{++}, pattern = 2.

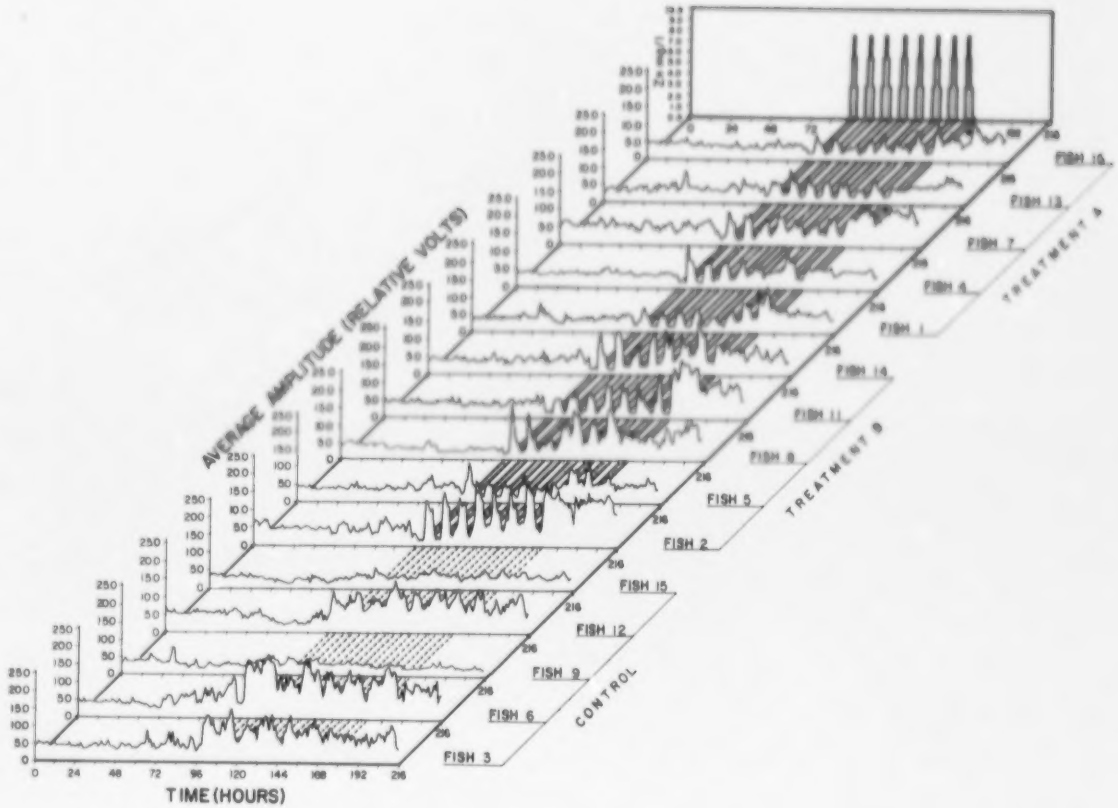

Figure C-3. Average ventilatory signal amplitudes for ventilatory test 3. Toxicant = Zn^{++}, pattern = 3.

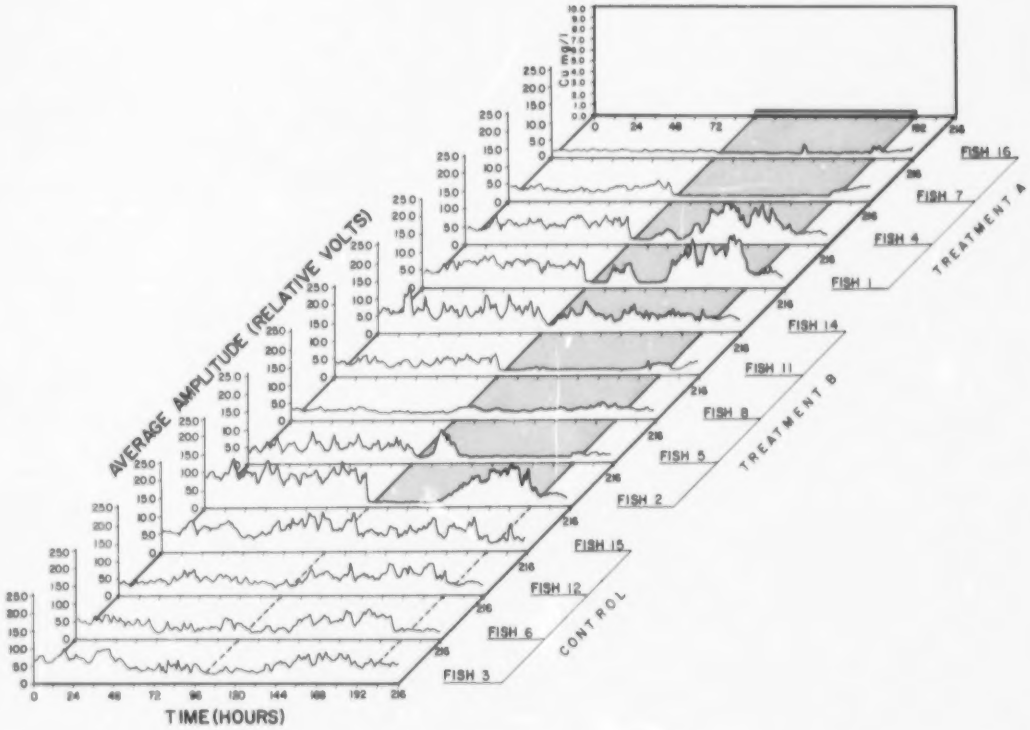

Figure C-4. Average ventilatory signal amplitudes for ventilatory test 4. Toxicant = Cu^{++}, pattern = 1.

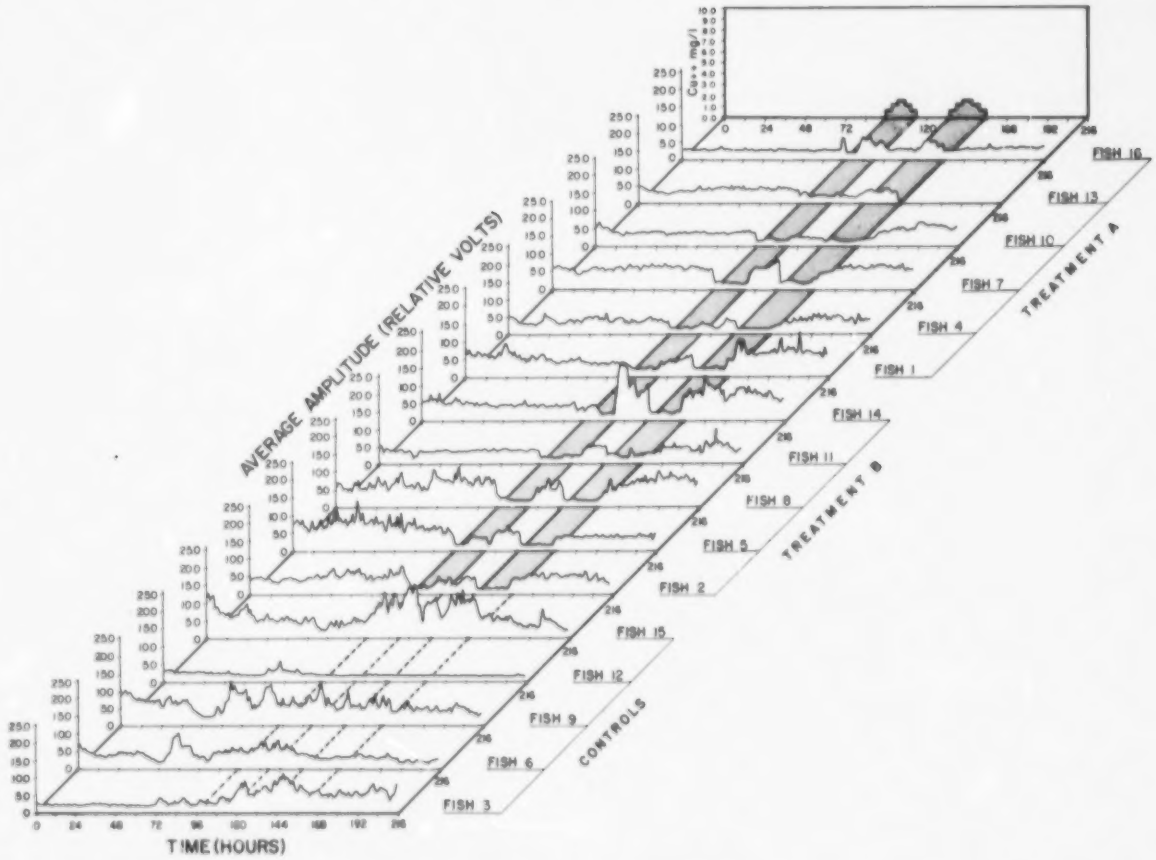

Figure C-5. Average ventilatory signal amplitudes for ventilatory test 5. Toxicant = Cu^{++}, pattern = 2.

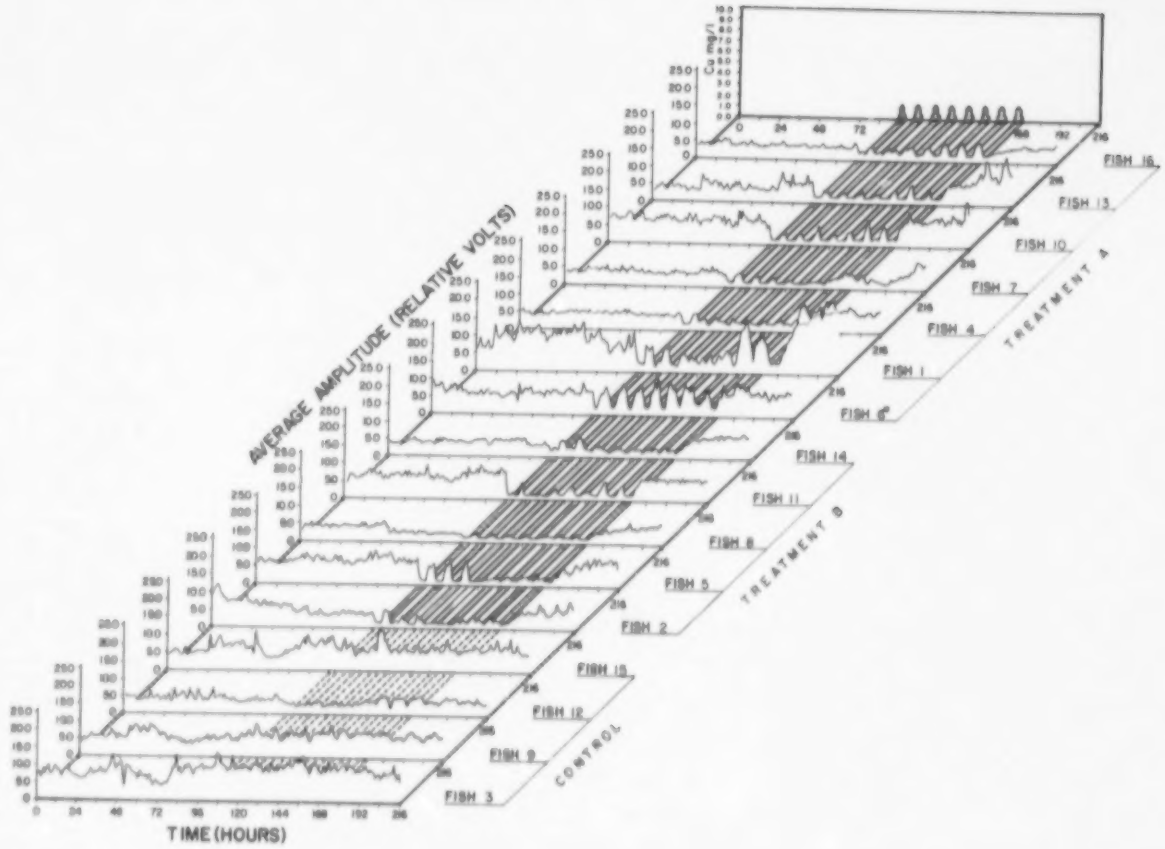

Figure C-6. Average ventilatory signal amplitudes for ventilatory test 6. Toxicant = Cu^{++}, pattern = 3. *Fish 6, a control, received a partial dose of toxicant due to water splash and was not included in the statistical analysis.

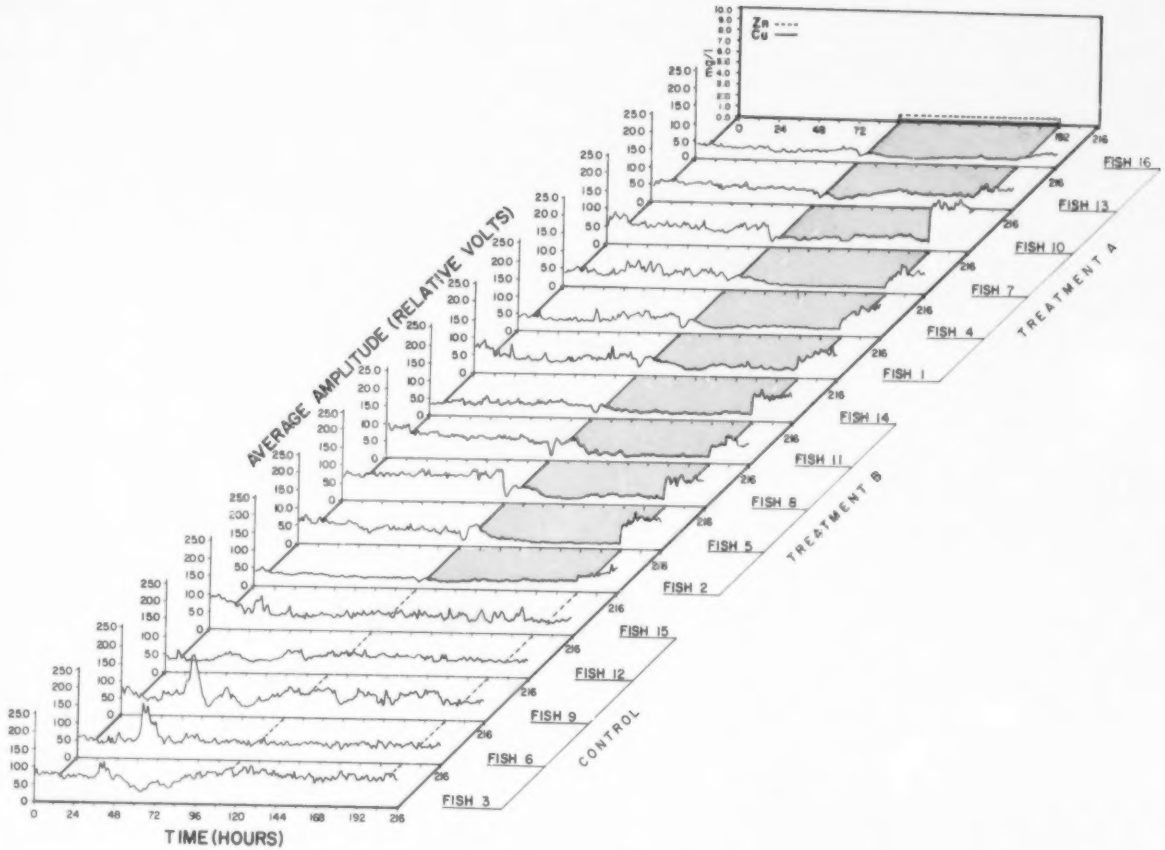

Figure C-7. Average ventilatory signal amplitudes for ventilatory test 7. Toxicant = MixA, pattern = 1.

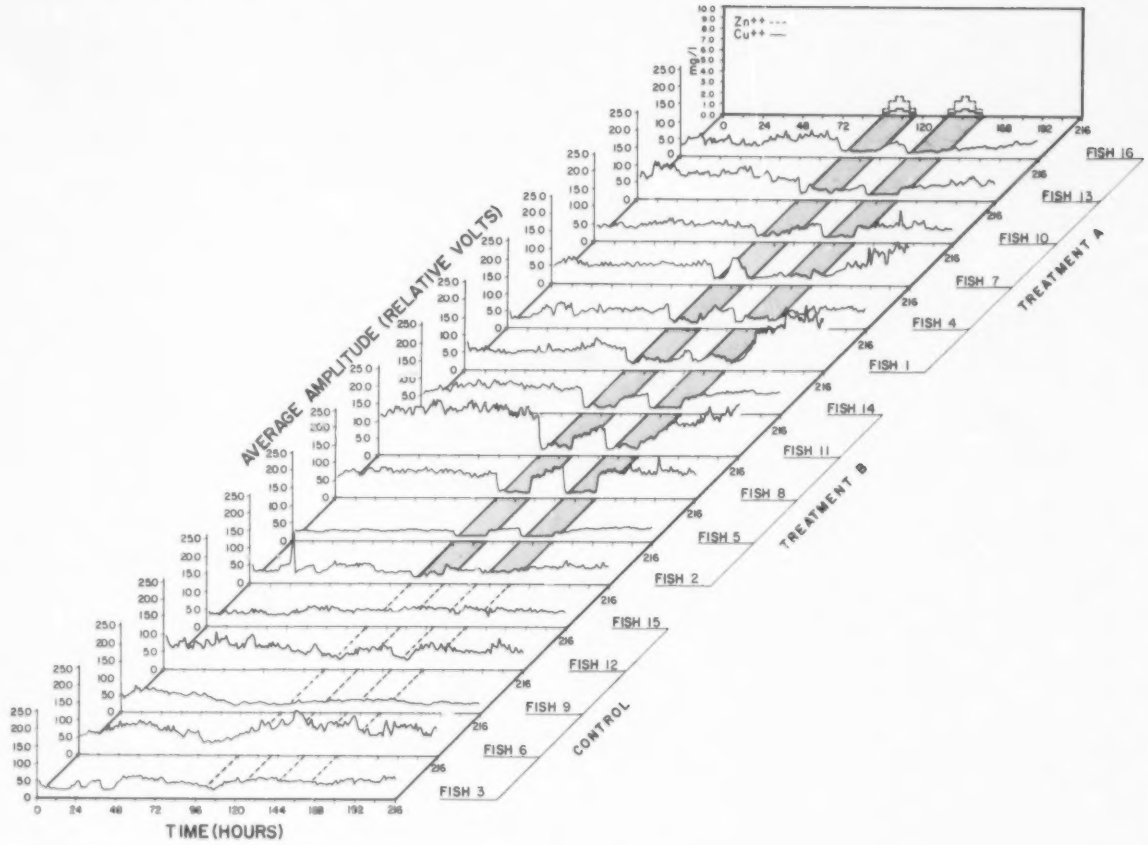

Figure C-8. Average ventilatory signal amplitudes for ventilatory test 8. Toxicant = MixA, pattern = 2.

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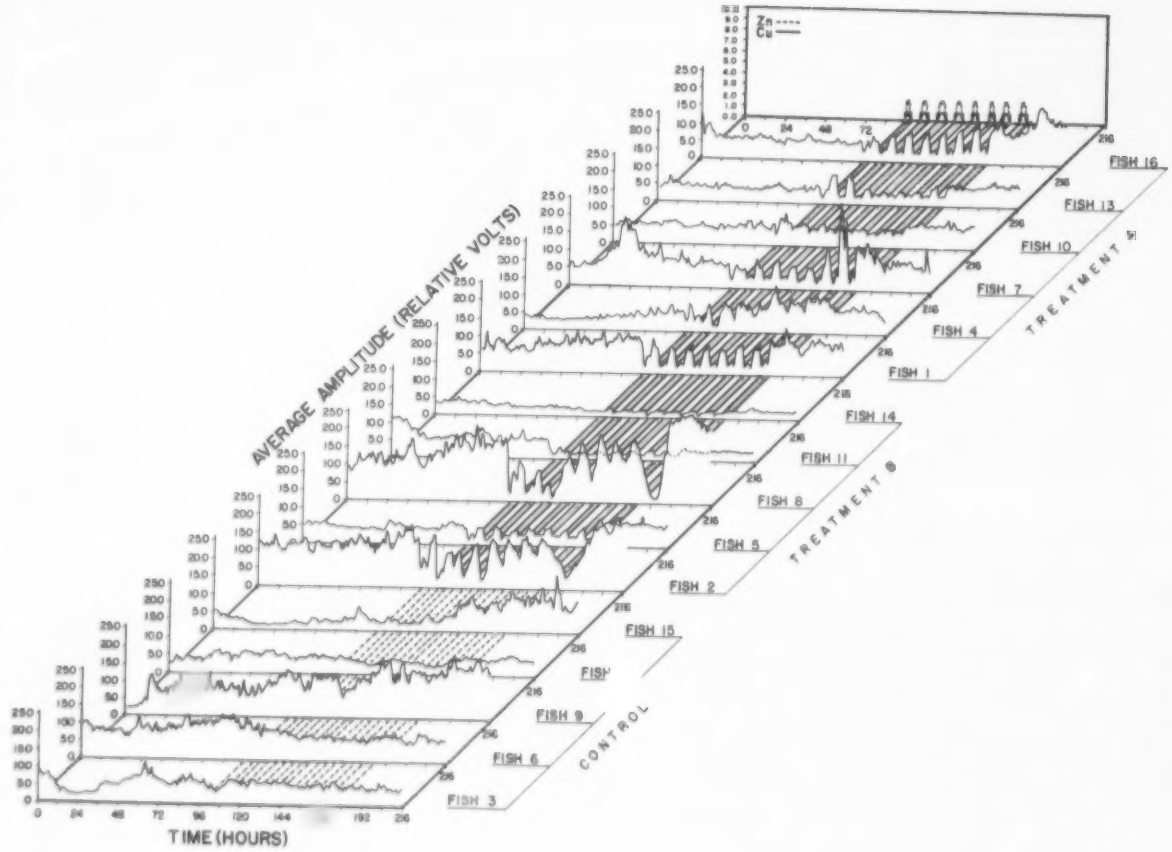

Figure C-9. Average ventilatory signal amplitudes for ventilatory test 9. Toxicant = MixA, pattern = 3.

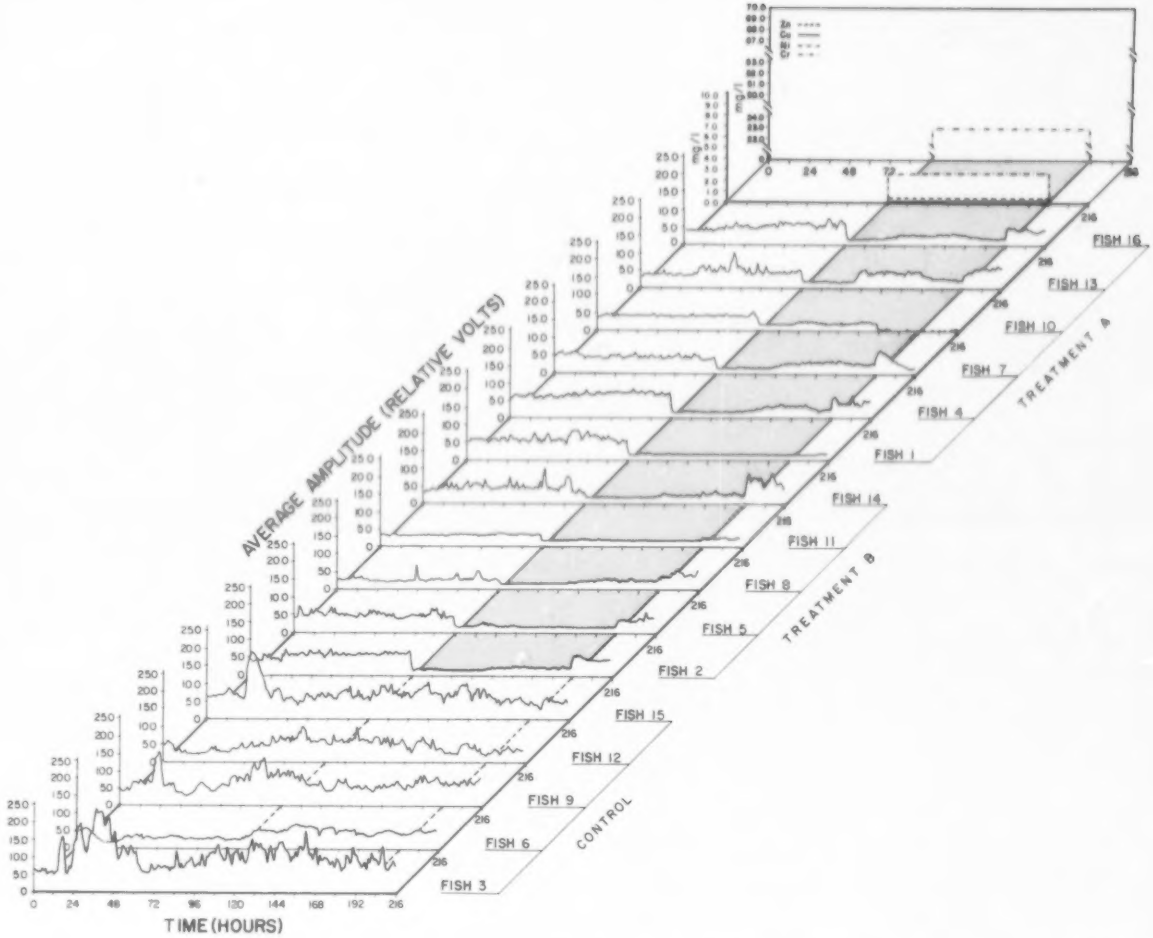

Figure C-10. Average ventilatory signal amplitudes for ventilatory test 10. Toxicant = MixB, pattern = 1.

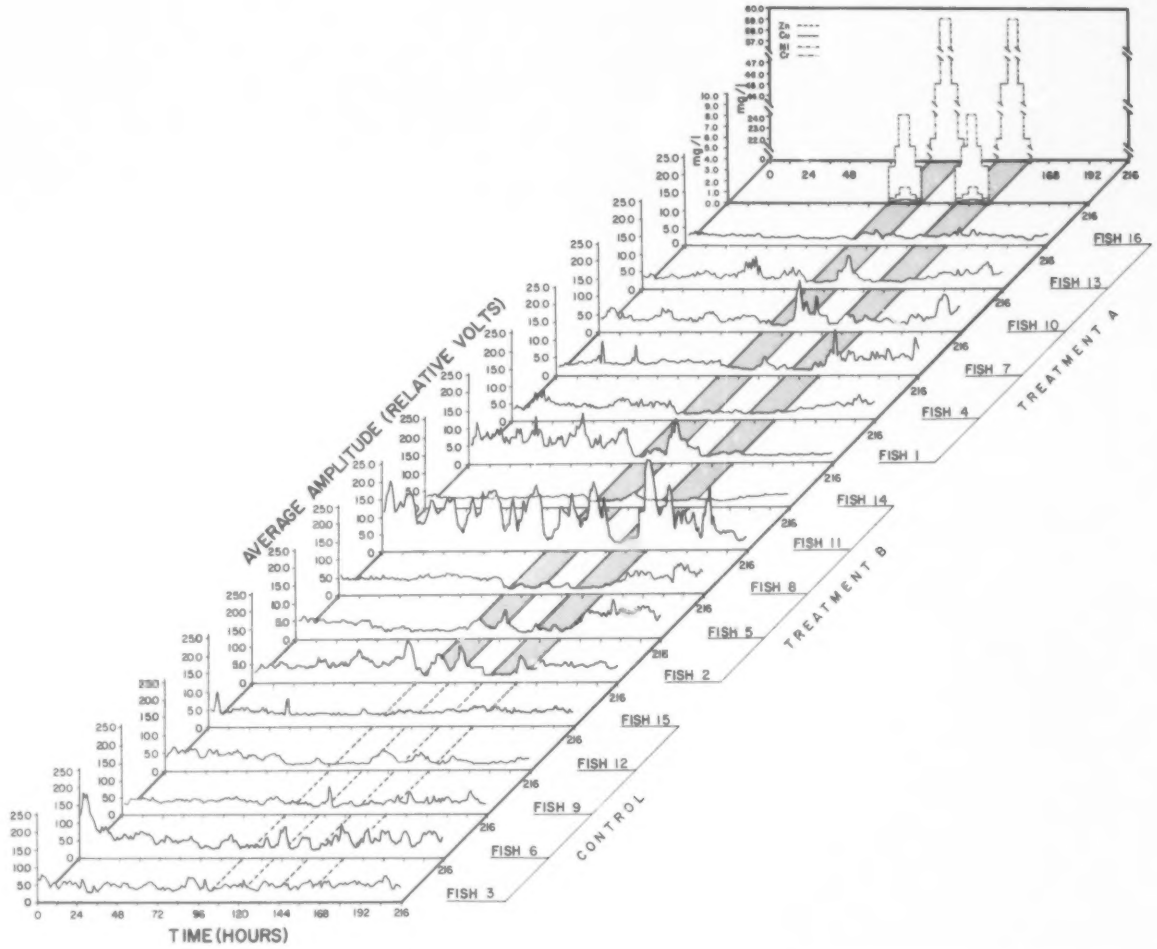

Figure C-11. Average ventilatory signal amplitudes for ventilatory test 11. Toxicant = MixB, pattern = 2.

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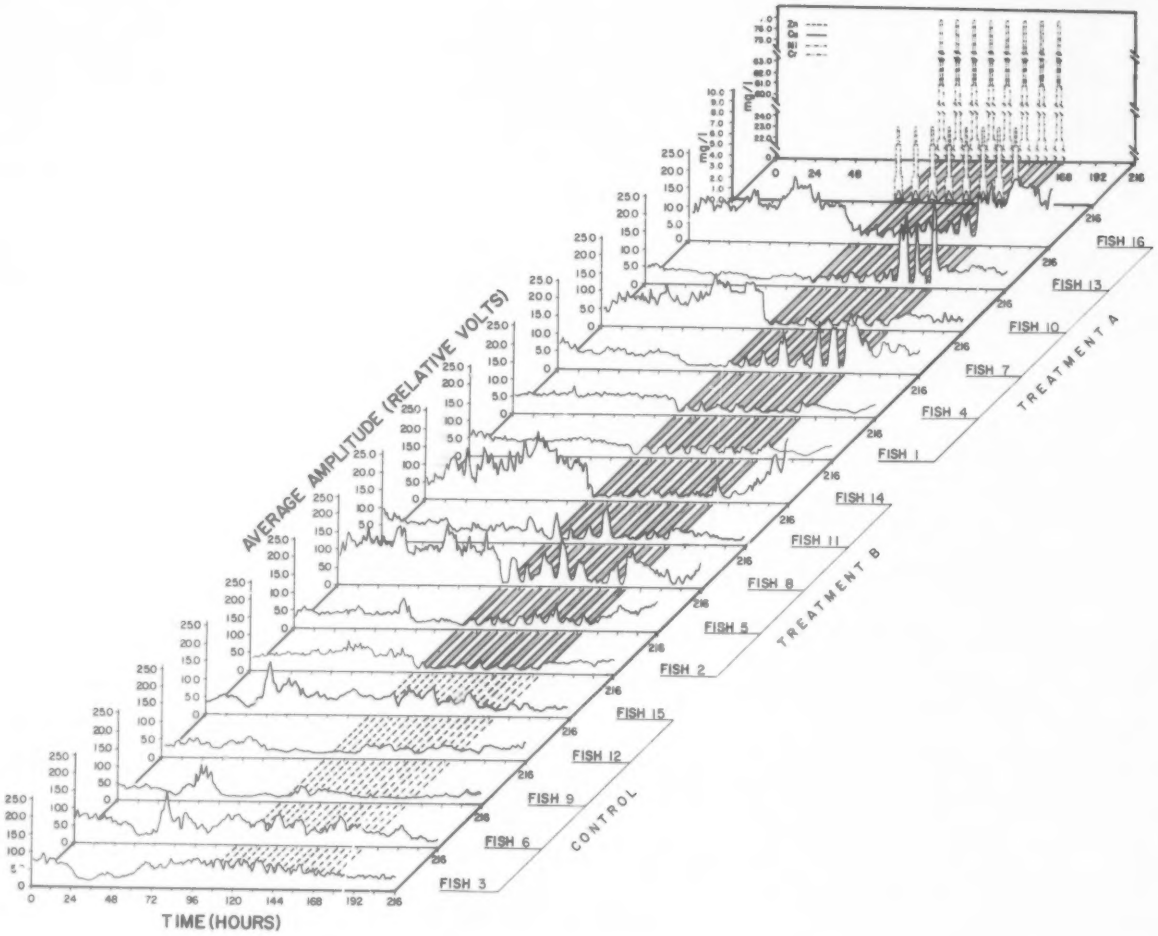

Figure C-12. Average ventilatory signal amplitudes for ventilatory test 12. Toxicant = MixB, pattern = 3.

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APPENDIX D

Examples of stressed and unstressed inter- and intra-individual variation as illustrated by strip chart recordings. These figures can be coordinated with the graphs of average ventilatory rate in Figure B-5 and the average signal amplitude in Figure C-5.

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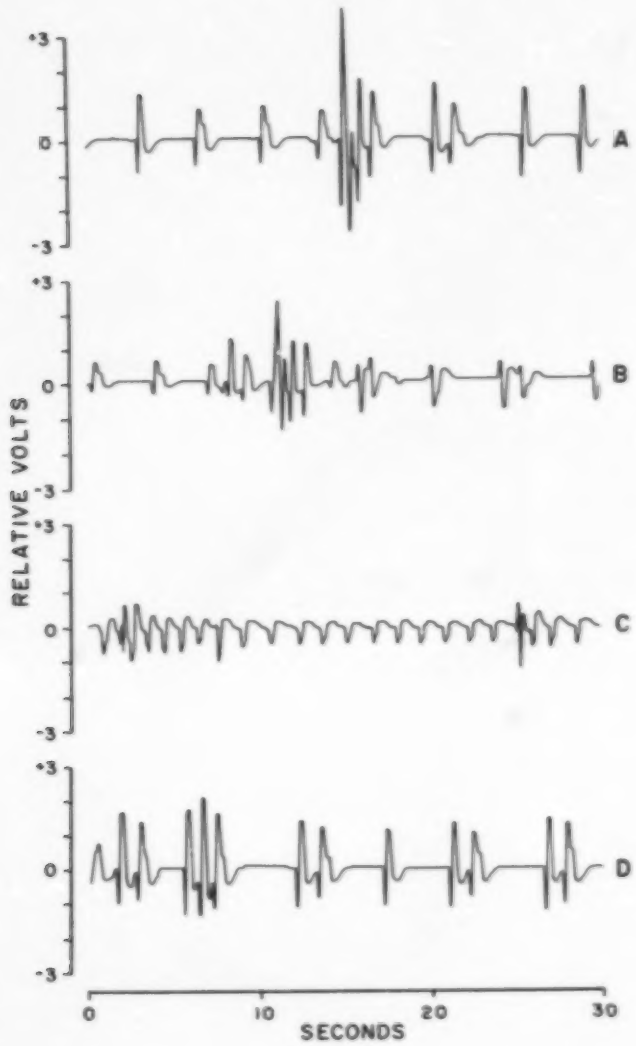

Figure D-1. Examples of the appearance of the ventilatory signals as they appear on a strip chart recorder. These recordings are for fish 6 (control) from run 5. The voltages indicated are arbitrary since the strip chart recorder further amplifies the signal that is being sent to the computer. The average rates and amplitudes recorded by the computer during the 15-min period from which these recordings were made and the total hours elapsed since the beginning of interval 2 are: A. 14 cts/min, 3.6 volts, 22.5 hr; B. 27, 2.8, 44.5; C. 49, 3.1, 74.5; D. 25, 5.3, 94.5.

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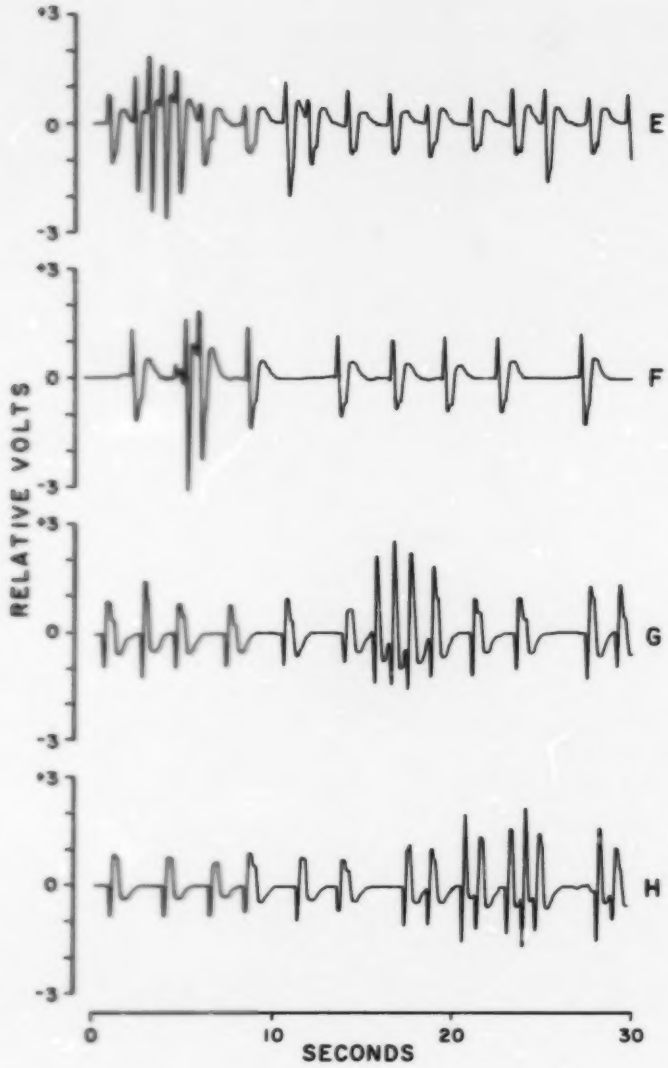

Figure D-1 (cont.). Ventilatory signals for fish 6 (control) from run 5. The computer values for the average rates and amplitudes and the elapsed time are: E. 35 cts/min, 6.7 volts, 94.5 hr; F. 25, 5.9, 97; G. 21, 5.8, 115.5; H. 31, 4.7, 123.

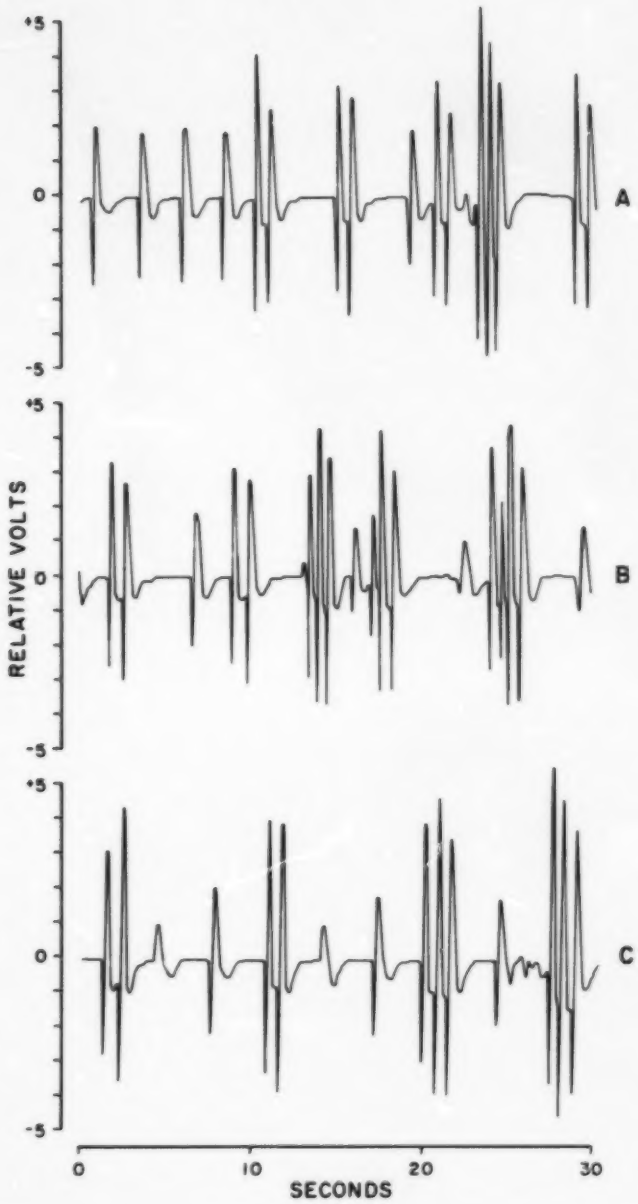

Figure D-2. Ventilatory signals for fish 5 (treated) from run 5. The computer values for the average rates and amplitudes and the elapsed time are: A. 25 cts/min, 6.2 volts, 22.5 hr; B. 25, 8.0, 44.5; C. 20, 6.5, 74.5.

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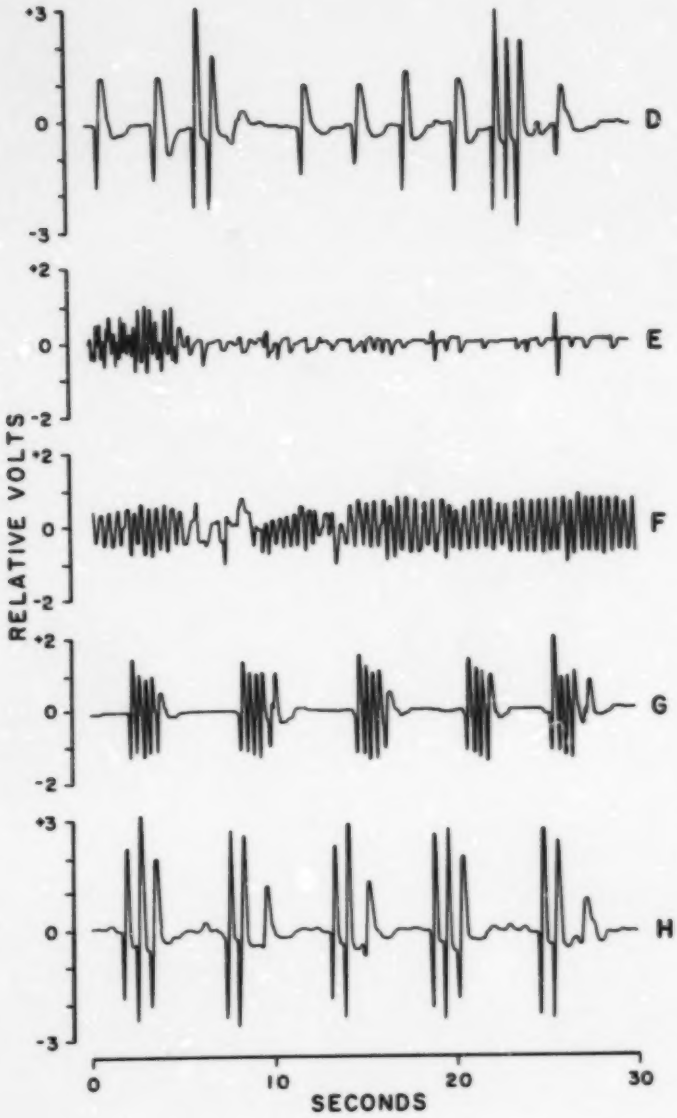

Figure D-2 (cont.). Ventilatory signals for fish 5 (treated) from run 5. The computer values for the average rates and amplitudes and the elapsed time are: D. 25 cts/min, 4.4 volts, 94.5 hr; E. 56, 1.8, 97 (after 1 hr of toxicant exposure, concentration = 0.6 mg/l Cu^{++}); F. 127, 3.8, 107.5 (after 11.5 hr of exposure, 1.9 mg/l); G. 32, 3.4, 115.5 (after 19.5 hr exposure, 0.6 mg/l); H. 42, 6.1, 123 (between toxicant applications after 8 hr of recovery time).

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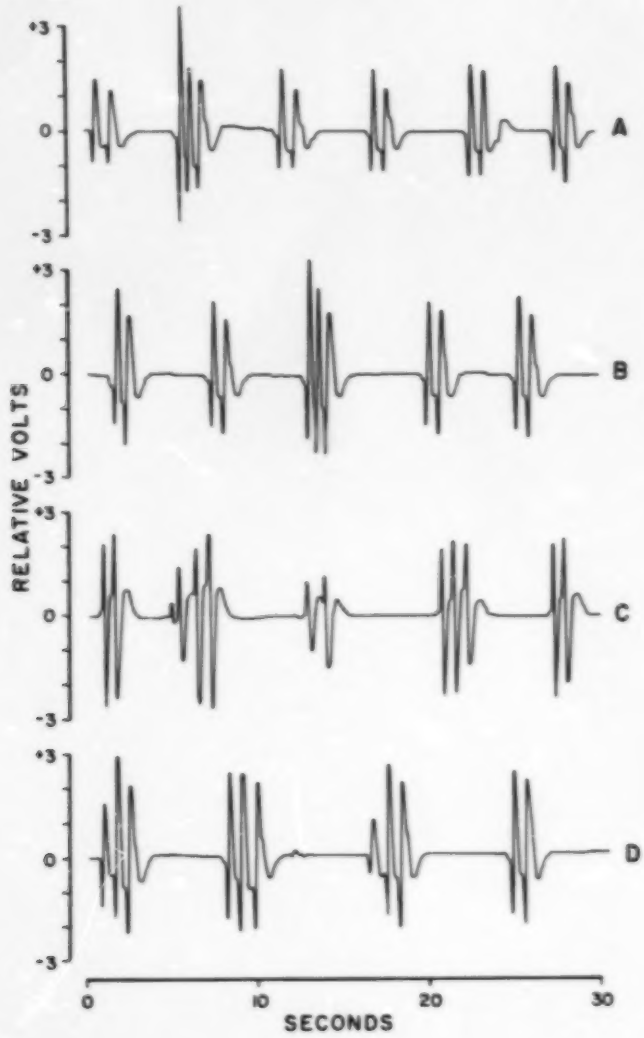

Figure D-3. Ventilatory signals for fish 7 (treated) from run 5. The computer values for the average rates and amplitudes and the elapsed time are: A. 18 cts/min, 6.1 volts, 22.5 hr; B. 18, 6.0, 44.5; C. 21, 6.3, 74.5; D. 20, 5.3, 94.5.

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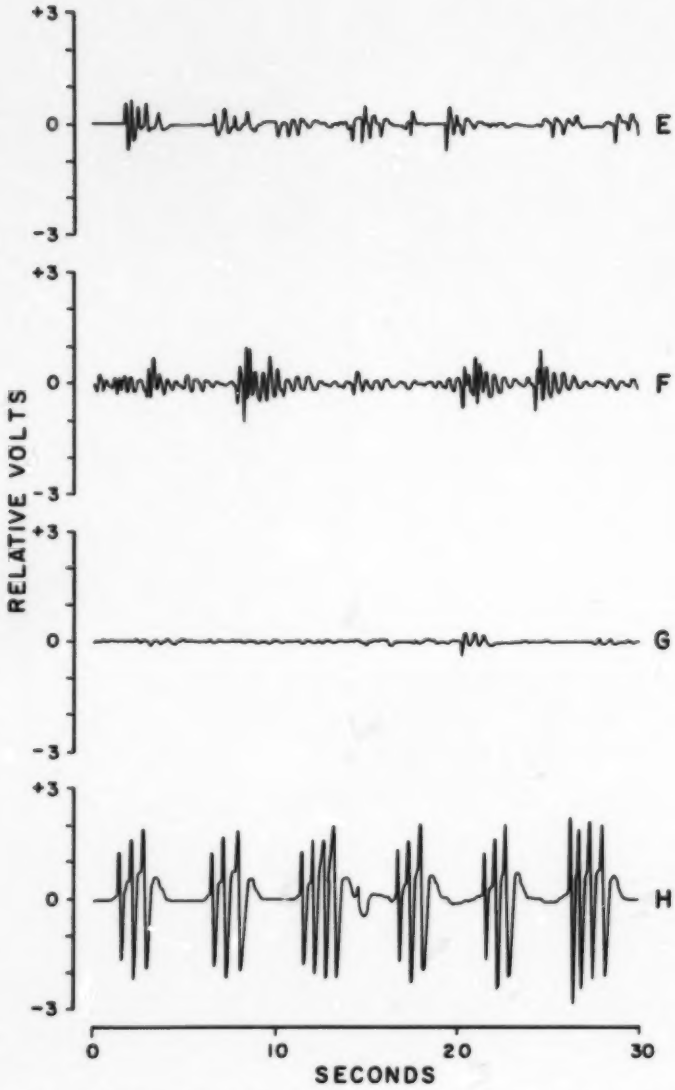

Figure D-3 (cont.). Ventilatory signals for fish 7 (treated) from run 5. The computer values for the average rates and amplitudes and the elapsed time are: E. 46 cts/min, 2.1 volts, 97 hr (after 1 hr of toxicant exposure, concnetration = 0.5 mg/l Cu^{++}); F. 75, 2.2, 107.5 (after 11.5 hr of exposure, 1.4 mg/l); G. 13, 1.7, 115.5 (after 19.5 hr exposure, 0.5 mg/l); H. 36, 6.7, 123 (between applications after 8 hr of recovery time).

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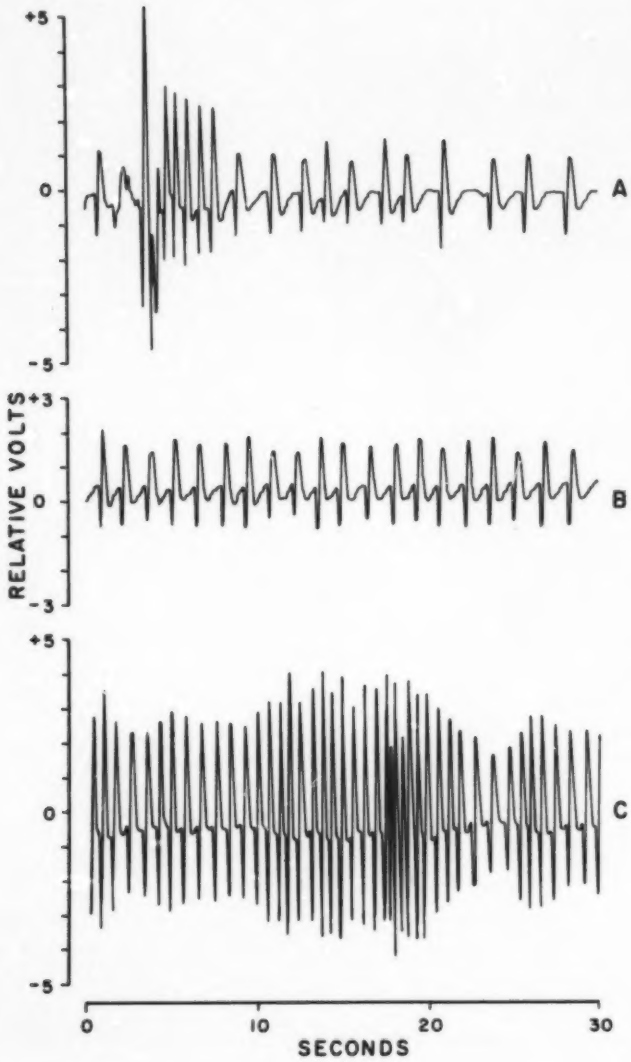

Figure D-4. Ventilatory signals for fish 8 (treated) from run 5. The computer values for the average rates and amplitudes and the elapsed time are: A. 37 cts/min, 7.7 volts, 22.5 hr; B. 40, 5.8, 44.5; C. 68, 13.9, 74.5.

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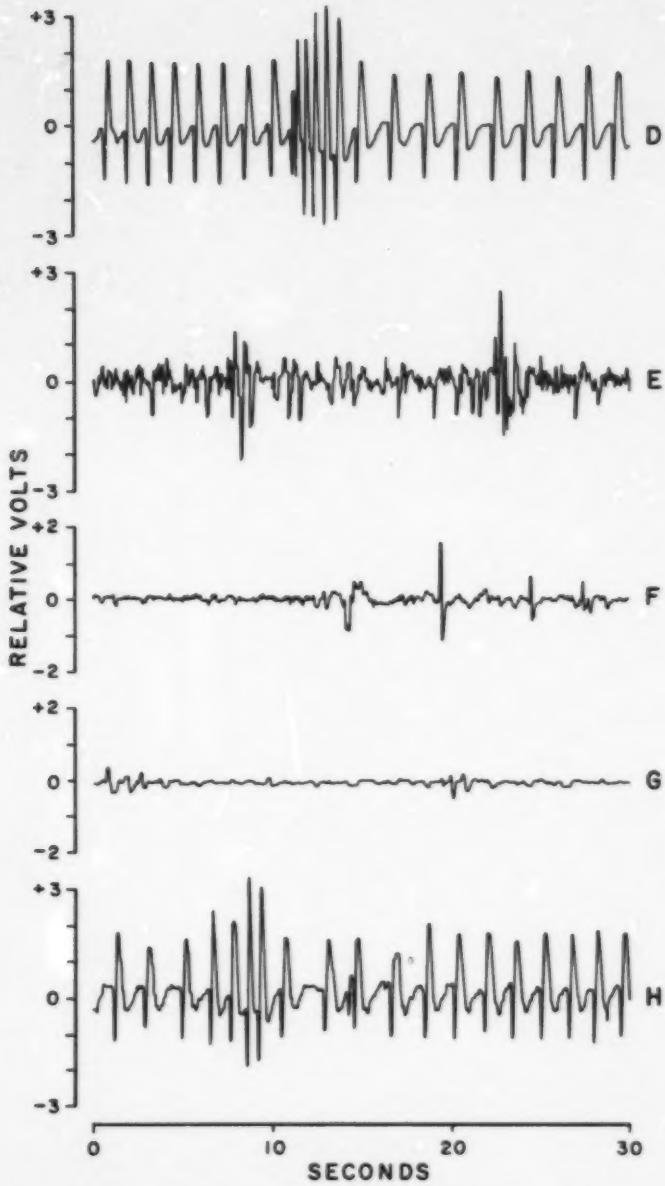

Figure D-4 (cont.). Ventilatory signals for fish 8 (treated) from run 5. The computer values for the average rates and amplitudes and the elapsed time are: D. 38 cts/min, 7.7 volts, 94.5 hr; E. 57, 3.6, 97 (after 1 hr of toxicant exposure, concentration = 0.5 mg/l Cu^{++}); F. 20, 1.8, 107.5 (after 11.5 hr of exposure, 1.3 mg/l); G. 26, 1.8, 115.5 (after 19.5 hr exposure, 0.5 mg/l); H. 44, 6.9, 123 (between toxicant applications after 8 hr of recovery time).

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